



Effect of hypoxia and acidification on the physiology of the Peruvian scallop *Argopecten purpuratus* (L.)

Rosa Cueto Vega

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HAL Id: tel-04416304

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THESE DE DOCTORAT DE

L'UNIVERSITE
DE BRETAGNE OCCIDENTALE

ECOLE DOCTORALE N° 598
Sciences de la Mer et du littoral
Spécialité : Biologie marine

Par

Rosa CUETO VEGA

Effect of hypoxia and acidification on the physiology of the Peruvian scallop *Argopecten purpuratus* (L.)

Thèse présentée et soutenue à Plouzané, le 27/01/2022

Unité de recherche : IUEM, Laboratoire des Sciences de l'Environnement Marin

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Remerciements

Tout d'abord, je tiens à exprimer ma profonde gratitude envers mes directeurs de thèse, Gérard, Fred, Patricia, et mon encadrant Jonathan. Leur patience, expertise, conseils avisés, et soutien, en particulier dans la phase finale de ma thèse, ont été précieux. Travailler avec eux a été une expérience exceptionnelle.

Je souhaite également remercier chaleureusement Luis Tito de Morais, ancien directeur de l'UMR LEMAR, ainsi que toute l'équipe du LEMAR qui m'a accueillie pendant mon séjour en France. Leur hospitalité et leur collaboration ont été inestimables.

Mes remerciements vont également à l'école doctorale et à l'UBO pour leur assistance précieuse dans les démarches administratives. Je tiens à exprimer ma reconnaissance envers Elisabeth Bondu de l'EDSM, pour son amabilité et sa volonté inébranlable d'aider.

Je tiens à exprimer ma gratitude envers les membres de mon jury de thèse, Ilie Racotta, Katherina Brokordt, Marcela Cantillanez, et Jorge Tam, ainsi qu'envers Arnaud Huvet et Piero Calosi, membres de mon comité de suivi individuel, pour leurs discussions et leurs critiques constructives sur mon travail.

Asimismo, me gustaría agradecer a mis colegas del IMARPE de los laboratorios de Ecofisiología Acuática, Alimento vivo y Oceanografía química que me permitieron trabajar con ellos y facilitaron la realización de la parte experimental de mi tesis: Arturo, Jhon, Mariandrea, Gheraldine, Wilson, Michelle, Juan Arguelles.

Gracias a mis estudiantes de licenciatura, Julio Fernando y Lucero que trabajaron conmigo durante la realización de los experimentos, fue un gusto enorme participar en su formación y luego poder compartir sus logros.

Muchas gracias a la Escuela Doctoral Franco-Peruana en Ciencias de la Vida de la UPCH, y a todas las instituciones peruanas y francesas que la respaldan, por su apoyo financiero durante la realización de mi tesis. Especialmente quiero agradecer al IRD en Perú, que a través de la coordinación de François Colas, fueron actores clave para la parte experimental de mi tesis y me facilitaron el intercambio entre Perú y Francia.

Gracias a mis colegas de la Facultad de Pesquería de la Universidad Nacional Agraria la Molina, por la confianza depositada en mí.

Finalmente, me gustaría agradecer a mi familia, gracias a mi mamá María, papá Marcial y a mis hermanos Eli, Nino, Raquel y Esther que a su manera siempre están escuchándome y apoyándome; a mi esposo Arturo y a mi hija Adriana, por su amor incondicional y compartir conmigo esta rica aventura de la tesis.

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Chapter 1

Introduction

1.1 The Humboldt current system: productive but stressful

The Humboldt current system (HCS) is one of the four major global Eastern Boundary Currents Systems (EBCS ; also called Eastern Boundary Upwelling Systems, EBUS) ; it sustains the most important production of small pelagic fishes in the world (Chavez et al., 2008). Located from approximately 42°S up to about the equator (Montecino et al., 2006), it is characterized by a dominance of northward winds, inducing an offshore Ekman transport and subsequently the upwelling of nutrient-rich, cold subsurface water (Hill, 1998). Besides, the HCS has a permanent, intense and shallow oxygen minimum zone (OMZ; Gutiérrez et al., 2008). Literature generally divides the HCS into two zones : the northern HCS (NHCS : 0-20°S ; Grados et al., 2018), mainly in Peru, and the southern HCS (SHCS : from 18-40 °S ; Thiel et al., 2007) in Chile. The former is mainly influenced by the Equatorial Current System, whereas the latter exhibits increased seasonality in both wind forcing and oceanic response. Nevertheless, the narrow continental shelf and meridionally oriented coastline of South America allow efficient transmittal of atmospheric and oceanographic signals between both zones (Thiel et al., 2007). Besides the latitudinal variability mentioned before, there is also a high temporal variability. For example, both interannual and interdecadal environmental variability scales have been detected in the NHCS (Colas et al., 2012 ; Echevin et al., 2008 ; Montes et al., 2011 ; Grados et al., 2018 ; Cardich et al., 2019). Between these different timescales, El Niño Southern Oscillation (ENSO) is the strongest natural climate fluctuation and occurs at interannual scales (eg., Wallace et al., 1998 ; Timmermann et al., 1999). During the warm ENSO events, warm water pool originating from the tropical Western Pacific approaches South America ; the northward flowing of the Humboldt current (which bring relatively cool waters) get weak and, as a consequence, the upwelling intensity decreases (Palma et al., 2006). It has been shown that a warm ENSO phase can positively affect some benthic organisms by stimulating their growth and reproduction (Arntz et al., 2006). In the northernmost part of the HCS, the consequences of these warm phases are negative ; increased rainfalls, floods, and changes in the distribution and abundance of fishery resources affect the livelihood of coastal populations (Arntz et al., 2006).

As mentioned before, the NHCS is associated with a strong oxygen minimum zone. Depending on years, sporadically advected OMZ water or high productivity associated with the coastal upwelling can lead to hypoxia in shallow coastal waters (Chavez et al., 2008). Generally, O₂ concentrations below 60 µmol kg⁻¹ (about 5% O₂ saturation) are considered as severe hypoxic conditions in ecological studies (Deutsch et al., 2011). Shallow coastal waters sustain a strong productivity with a net production of oxygen by photosynthesis. By contrast, degradation processes of organic matter below the photic zone consume rapidly dissolved oxygen (Stramma et al., 2010). This natural pattern is exacerbated due

to anthropogenic causes (e.g. nutrient over-enrichment of the waters as a result of pollution), and it has generated a global expansion of dead zones in coastal waters (Diaz and Rosenberg, 2008 ; Stramma et al., 2010). Dead zones and their interaction with expanding OMZs represent a global threat for coastal ecosystems and fisheries (Stramma et al., 2010, Fig. 1.1). Additionally, in the absence of oxygen, bacterial anaerobic degradation of organic matter may occur followed by the production of toxic sulfide (H_2S), that may cause massive mortalities in benthic and pelagic populations (Vaquer-Sunyer and Duarte, 2010).

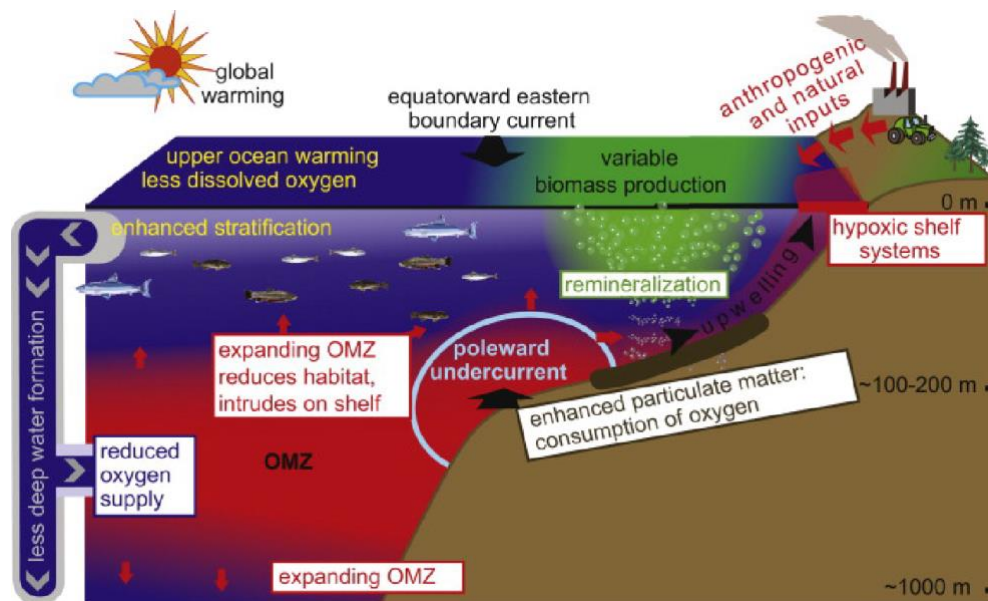


FIG. 1.1: Schematic of interactions between open ocean oxygen minimum zones with hypoxic shelf systems and dead zones on continental shelves of eastern ocean boundaries (from Stramma et al., 2010).

Most oceanographic investigations in the HCS have focused on zones relatively far from the coast until now (eg., Graco et al., 2017 ; Altieri and Diaz, 2019). However, the studies on coastal ecosystems have increased in the last decade. For example, Mayol Fajardo and Castilla (2012) showed that the Chilean HCS suffers from ocean acidification and hypoxia, which compromises the health of marine biota. Studies in coastal shallow waters in Peru have shown intense and prolonged hypoxia affecting mainly benthic organisms that inhabit Peruvian bays (Aguirre-Velarde et al., 2016, 2019b). The last authors focused on the study of *Argopecten purpuratus*, a marine bivalve of commercial importance, and carried out high-frequency environmental monitoring and biological monitoring of *A. purpuratus* during a period of seven months in Paracas Bay. They evidenced highly variable conditions especially in Summer, with cold water bodies entering the bay, presumably due to the adjacent upwelling activity. This period was characterized by abrupt variations in temperature (up to 8 degrees in a few hours) and low oxygen saturations with concentrations that sometimes dropped close to anoxia. The environmental conditions were very harsh during several weeks, with events of turquoise watercolor, presumably due to anoxia and sulfides.

Future modeling scenarios (Representative Concentration Pathway 8.5, RCP8.5) to the next century predict an intensification of upwelling in EBUS at high latitudes (eg. for the California Upwelling System) whereas wind and upwelling intensity (or extension) will probably decrease at low latitudes (eg.

HCS) (Bindoff et al., 2019, and references herein). The latter, along with ocean deoxygenation, acidification, thermal stratification and warming (García-Reyes et al., 2015), could lead to unprecedented ecological and socio-economic effects in the Humboldt Current System (Bopp et al., 2013 ; Oyarzún and Brierley, 2019).

1.2 The Peruvian scallop, *Argopecten purpuratus*

1.2.1 Systematic position

Argopecten purpuratus (Lamarck, 1819), known as the Chilean or Peruvian scallop, and named in Spanish either “concha de abanico” (in Peru) or “ostión del norte” (in Chile), belongs to the Pectinidae family. This species was originally described as *Pecten purpuratus* (Lamarck, 1819). Its taxonomic position is detailed below :

Phylum	<i>Mollusca</i>
Class	<i>Bivalvia</i> (Linné, 1758)
Family	<i>Pectinidae</i> (Rafinesque, 1815)
Genus	<i>Argopecten</i> (Monterosato, 1889)
Species	<i>purpuratus</i> (Lamarck, 1819)

1.2.2 Biology and ecology

This epibenthic species is mainly found in shallow waters from 2 to 40 m water depth in semi-protected bays on sandy, stony or sandy-muddy substrates (Wolff and Mendo, 2000). Its geographic distribution ranges from northern Peru (Paíta, 5°81'S, 87°11'W) to central Chile (Tongoy, 37°S, 71°W, Fig. 1.2 ; Marincovich, 1973). Due to relocation for aquaculture purposes, it can be found up to Los Organos, Peru (4.17°S) in the North (Mendo et al., 2016). The main natural beds in Peru are located in Sechura Bay (5.7°S), Lobos de Tierra Island (6.4°S), and Independence Bay (14.25°S ; Mendo et al., 2008). Main beds along the Chilean coast are in Tongoy (30°S ; Stotz and Gonzalez, 1997), Mejillones (23.1°S) and La Rinconada (23.5°S) Bays (Avendaño and Le Pennec, 1997 ; Avendaño et al., 2017). Scallop populations from Peru and Chile (between 5°S and 23°S) have been shown to present high genetic diversity but no genetic structure with latitude (Marín et al., 2013 ; Acosta-Jofré et al., 2020).

This scallop species inhabits areas presenting a wide range of water temperature (between 12 and 25°C), showing a high thermal acclimatization ability (Navarro et al., 2000). Wolff (1987) suggested that *A. purpuratus* was a relict form of the subtropical fauna which once inhabited Chile and Peru, retaining characteristics of a warm-water species even after the general cooling of the oceans at the end of the Miocene (see Avendaño et al., 2017, for review of the impacts of unusual warming events such as ENSO events on populations of this scallop species). Salinity tolerance may be much lower than temperature tolerance since the Peruvian scallop is found in waters with salinities ranging between 33 and 35 PSU (Osorio y Piwonka, 2002). This species seems relatively well adapted to living in low oxygen concentrations: Aguirre-Velarde et al. (2016) classified this species as a good oxy-regulator under broad oxygen levels at both 16°C and 25°C, showing little effect on metabolism above dissolved oxygen saturation (DO_{sat}) of 24.4%.

In Peru, major scallop predators are marine snails, such as *Thais chocolata*, crabs (*Cancer porteri*, *Cancer setosus* and *Cancer polyodon*), sea stars (*Luidia* sp.), octopuses (*Octopus mimus*), and rays (*Myliobatis* sp.) (Mendo et al., 2016).

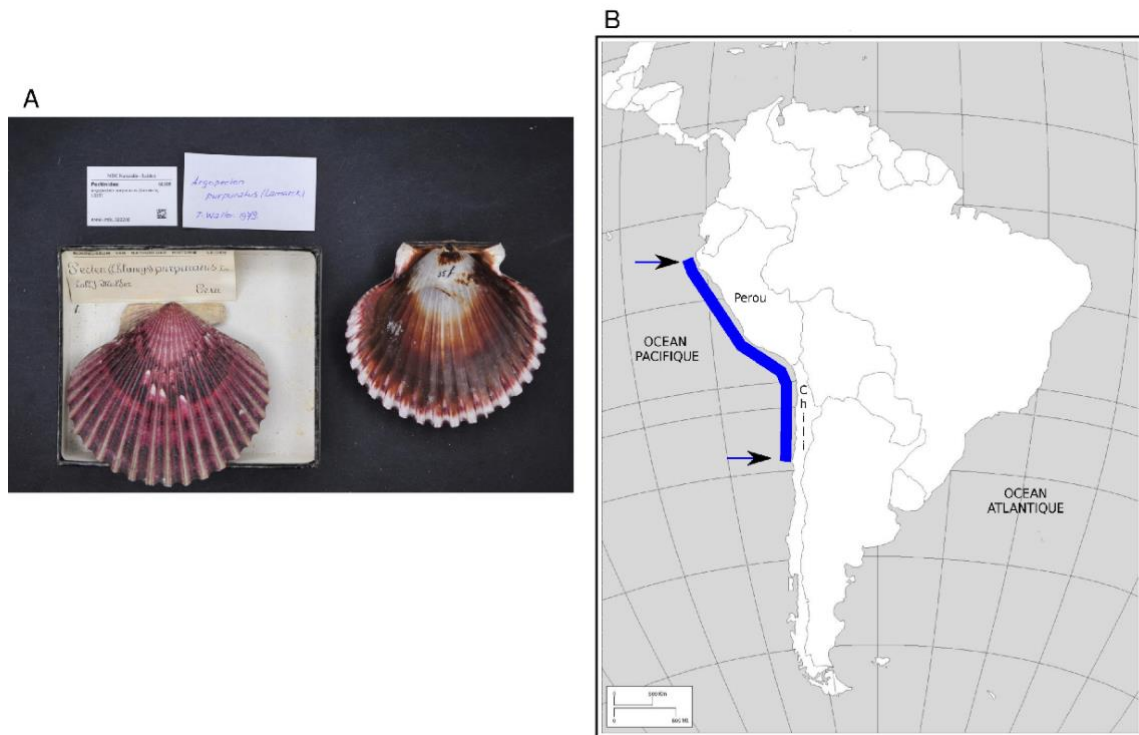


FIG. 1.2: (A) *Argopecten purpuratus* shells (from NBC-Leiden, 2015) and (B) geographic distribution (from González et al., 2002).

A. purpuratus is a filter-feeder and may feed on phytoplankton, zooplankton and detritus (Rouillon et al., 2002 ; Mendo and Wolff, 2003). It has been shown that diatoms contribute around 80% of the total abundance of the ingested phytoplankton species (Rouillon et al., 2002). The species in Peru has an asymptotic shell growth with maximum height ranging between 99 and 140 mm depending on scallop beds (Wolff and Mendo, 2000). Growth of the Peruvian scallop is known to vary with latitude, water depth, scallop density, and environmental conditions ; for example, there are some records of increased growth rate during strong El Niño warming events (Mendo et al., 2016). Previous studies in Chile also showed the strong impact of warm temperature on growth of scallop postlarvae, juveniles and adults (Cantillán-Silva et al., 2007 ; Avendaño and Cantillán, 2008 ; Thébault et al., 2008). *A. purpuratus* exhibits daily shell microstriae formation in Peru, although this periodicity may vary according to some environmental variables (Aguirre-Velarde et al., 2015).

The species is a functional hermaphrodite with a spawning asynchrony between individuals (Avendaño and Le Pennec, 1997 ; Cantillanez et al., 2005). The individuals exhibit a continuous production of gametes, with partial or complete spawnings along the year (Chavez and Ishiyama, 1989 ; Avendaño and Le Pennec, 1996 ; Avendaño and Le Pennec, 1997 ; Cantillanez et al., 2005). Spawning activity is more intense between September and May (Wolff, 1988 ; Avendaño and Cantillán, 2008). Maturity is reached at a shell height of 25 mm (Mendo Aguilar et al., 1989). Individual fecundity is directly related to size:

specimens of 60 and 90 mm shell height may produce around 6 millions and 28 millions of eggs, respectively (Avendaño et al., 2001). Spawning was shown to be correlated with the lunar cycle: spawning occurs approximately 1 week after the new moon (Cueto-Vega, 2016 ; Aguirre-Velarde et al., 2019b).

1.2.3 Fishery and Aquaculture

Peruvian scallops are the second most caught mollusk species in Peru, after the Humboldt squid *Dosidicus gigas* (Mendo et al., 2016). During the year 2020, scallop catches accounted for 8.4% of the total production of mollusks in Peru (PRODUCE, 2020). Scallop catches peaked in 2010, 2011 and 2014 with 62 827, 93 050 and 56 002 metric tons, respectively (Fig. 1.3). Between 2015 and 2018, abrupt drops in catches affected the scallop fishery, but catches in 2019 were almost equal to the ones in 2010 and 2014 (Fig. 1.3).

A. purpuratus is also one of the most important aquaculture species in the country. For example in 2020, it was ranked in second place after trout (with 31.1 and 37.7% of total aquaculture production, respectively ; PRODUCE, 2020). There are 384 marine concessions along the Peruvian coast for the cultivation of Peruvian scallops (PRODUCE, 2020), the most productive areas being located in bays from Piura (~5°S) to Ancash (~9°S). Scallops seeds (i.e. individuals of 25-40 mm shell size) are placed in the concessions and cultured until a size >65 mm in 7 to 18 months depending on site and culture system (Mendo et al., 2008, 2016). Bottom cultivation is the most common technique compared with suspended culture despite having lower growth rates in this system, because it is cheaper and allows a larger cultivation area (Alcázar and Mendo, 2008). After reaching a suitable size, scallops are collected and processed. The processing begins with the removal of shells, then muscles and gonads are frozen and packed. The aforementioned activities are performed by different operators (Sánchez Cuéllar, 2017). For example, in Sechura Bay (Piura), the scallop aquaculture production supports 5000 artisanal fishermen (in scallop farmer cooperatives), but also generates incomes for about 20 000 additional workers whose activities rely on scallop processing chains and associated businesses (Kluger et al., 2019a).

Most of the scallop production is exported as frozen products ; the rest is sold mainly fresh in the national markets (range 149-2000 t in the period 2011-2020 ; PRODUCE, 2020). In 2020, the main export destinations were the United States (39.9%), France (26.5%) and Spain (10.3% ; PRODUCE, 2020).

The scallop resource is vulnerable to exploitation (eg., Mendo et al., 2008, 2016 ; Avendaño et al., 2017), to extreme environmental events (eg., Aguirre-Velarde et al., 2019b ; Cueto-Vega et al., 2021), and to warm (El Niño) and cold (La Niña) periods (eg., Wolff, 1987 ; Wolff and Mendo, 2000; Cantillanez et al., 2005 ; Avendaño et al., 2008), which trigger regional and temporal fluctuations in abundance. Nevertheless, the lack of regular environmental and scallop monitorings (including stock assessment) leads to weak application of fishing regulations for this species (eg., Mendo et al., 2008, 2016 ; Avendaño et al., 2017).

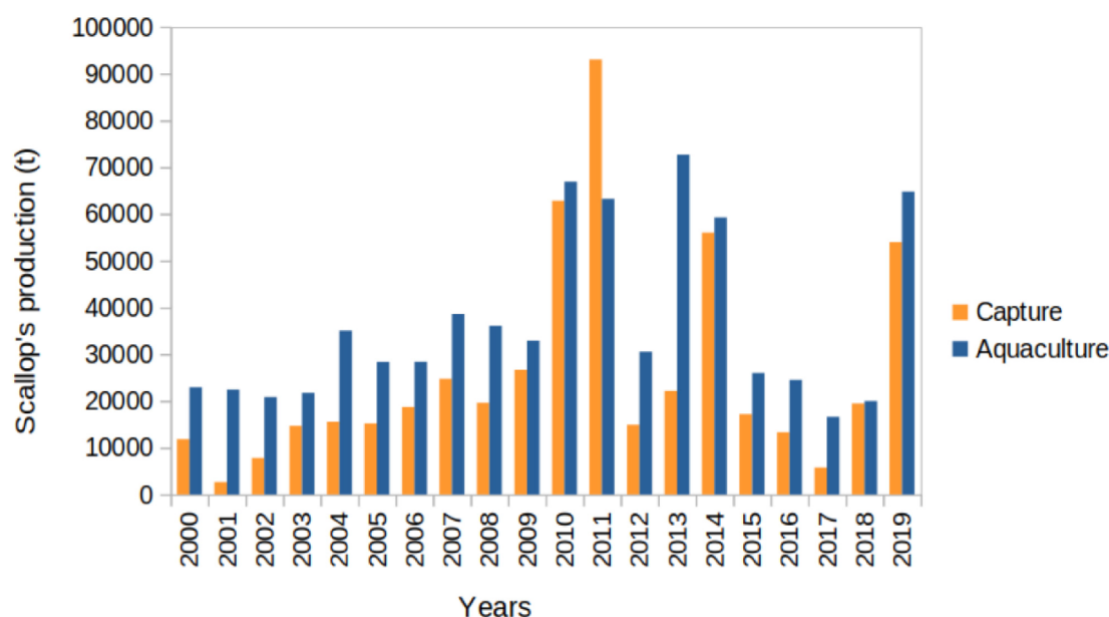


FIG. 1.3: Captures (landings) and Aquaculture production (metric tons) of *A. purpuratus* in Peru between the years 2000 and 2019 (from FAO, 2020 : <https://www.fao.org/fishery/statistics/>)

1.3 Ecophysiological approaches to assess bivalve responses to extreme environmental conditions

Physiological ecology or ecophysiology aims to explore how animals respond to their environment ; for this discipline, it is common to use experimental or modeling tools to assess and explain the physiological responses of the organisms to one or several environmental stressors. Organism's response can be assessed at the organism, cellular or molecular level.

1.3.1 Organism level

At the individual level, the study of physiological thresholds can provide valuable information on how the “body” can respond to its environment. Physiological thresholds have been used for example to explain the concept of “oxygen- and capacity-limited thermal tolerance” (OCLTT) proposed by (Pörtner and Lannig, 2009). The OCLTT concept addresses the molecular to whole-animal mechanisms that define thermal constraints on the capacity for oxygen supply to the organism in relation to oxygen demand (Pörtner et al., 2017). The thresholds (applied to temperature) proposed by these authors were the optimum, pejus (Tp), critical (Tc) and lethal levels (Td). In the optimum range, the performance (e.g., exercise, growth, reproduction) of organisms is maximal. In the pejus threshold, the organism's performance is getting worse due to the effort made by the animal to maintain its homeostasis. The following thresholds proposed by the authors are critical values (for cooling or warming) : if the organism surpasses these levels, oxygen availability becomes insufficient and the organism starts using its anaerobic metabolism. When surpassing the extreme threshold, the individual is no longer able to survive ; Pörtner (2010) called this threshold “denaturation temperature” (Td) ; it is characterized by the onset of damage to cells and proteins. For *A. purpuratus*, this Td should close to

the upper lethal temperature ($29 \pm 3^\circ\text{C}$; Urban, 1994). If dissolved oxygen levels are considered, probably the already known threshold could be the pejus level with its equivalent to the critical oxygen saturation (24% according Aguirre-Velarde et al., 2016). The other oxygen thresholds remain unknown.

To analyze the response at individual level, it is also important considering the species life cycle, because the ability to cope with environmental stressors may be greater in juveniles but can be weakened in early stages and in the spawners (Pörtner et al., 2014). In some bivalves, additional energy mobilization towards reproduction may affect tolerance to hypoxia ; thus, spawners (mature scallops) may be more vulnerable than immature individuals (Brokordt et al., 2015 ; Salgado-García, 2020). Besides, decreasing size has been proposed as an universal outcome to global warming (Daufresne et al., 2009 ; Gardner et al., 2011 ; Baudron et al., 2014). Small sizes have been also proposed as an advantage for aquatic organisms to cope with hypoxic conditions (Robb and Abrahams, 2003). Among bivalves, the ability to tolerate hypoxia may vary with size (Wang and Widdows, 1991 ; Shumway et al., 1983 ; Seto et al., 2004 ; Hicks and McMahon, 2005). This trend may be related with energy supply and demand : the “surface to volume ratio” hypothesis proposes that the energy supply is related with body surfaces (through gills, L^2) whereas energy demand has a component related with body volume (weight, L^3). This hypothesis has been presented and discussed in some studies carried out with mussel larvae and fish (Wang and Widdows, 1991 ; Robb and Abrahams, 2003). Currently there is a theory that mentions that the surface area of the gills (or other respiratory surfaces) of water-breathing ectotherms cannot, as two-dimensional structures, supply them with sufficient oxygen to keep up with the growth of their three-dimensional bodies (Pauly, 2021). Thus, according to the author a lower relative oxygen supply induces sexual maturation, and later a slowing and cessation of growth.

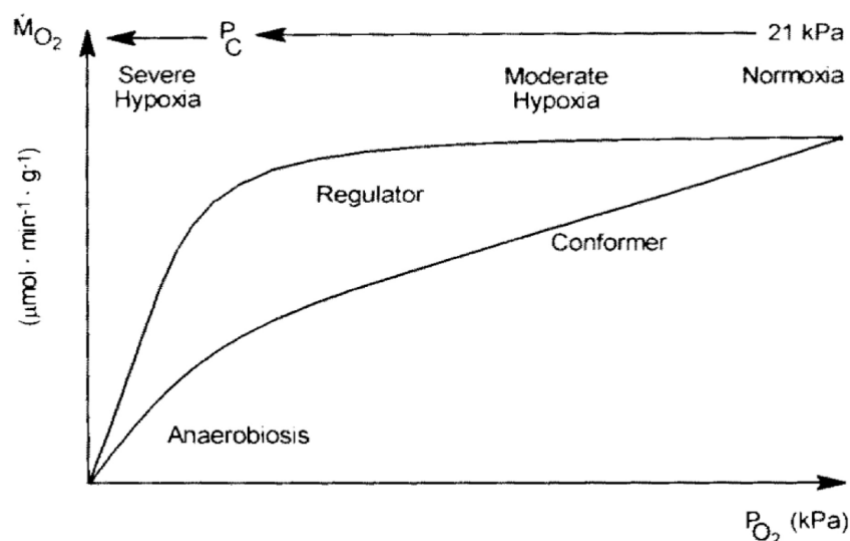


FIG. 1.4: General scheme of oxygen consumption (\dot{M}_{O_2} , $\text{mol}^{-1}\text{min}^{-1}\text{g}^{-1}$) at different ambient partial pressures of oxygen (p_{O_2} , kPa). Moderate hypoxia is compensated for oxygen supply by physiological mechanisms, whereas anaerobiosis starts during severe hypoxia and anoxia below the critical P_{cO_2} (from Grieshaber et al., 1992).

At individual-level, measuring the respiration rate under progressive hypoxia allows to describe how an organism is able to regulate its respiration. The ability to regulate oxygen consumption (respiration) depends on the hypoxia's intensity and duration, and may vary between species (Pörtner and Grieshaber, 1993). Some organisms are able to maintain a relatively constant oxygen consumption rate over a wide range of oxygen concentrations : they are called "oxy-regulators". Others have an oxygen consumption that depends on the oxygen concentration in the environment : they are called "oxy-conformers" (Grieshaber et al., 1992). Nevertheless, even oxy-regulators behave like oxyconformers below a critical threshold in partial pressure of oxygen called oxygen critical point (P_{cO_2} , see Fig. 1.4). This threshold coincides with the establishment of anaerobic metabolism (Pörtner and Grieshaber, 1993). Out of these two extreme cases, intermediate responses are frequently observed and most organisms exhibit a two-phase response with an oxygen regulatory phase under moderate hypoxia and an oxygen conformer phase under severe hypoxia. Both phases are separated by the P_{cO_2} . Because organisms differ in their P_{cO_2} , this value can be used for comparison purposes (Pörtner and Grieshaber, 1993). Organism's ability to regulate its oxygen consumption can be estimated through the percentage of oxygen regulation, by using the area beneath the "respiration rates vs oxygen percentage" curve (Hicks and McMahon, 2002). The last authors classified the oxygen-regulation capacity of the Brown Mussel *P. perna* as : excellent ($\geq 90\%$), good (80–89%), moderate (70–79%), and poor (60–69%), while the percentages lower than 60% indicate no regulation.

1.3.2 Cellular and molecular levels

Cellular and molecular approaches may help to identify the types of lethal and sublethal stresses that account for observed changes in distribution patterns, and to characterize the capacities of organisms to acclimatize to environmental changes (Somero, 2012). By using the thresholds mentioned in the previous section and the molecular response of the organism, it may be possible to know the degree of acclimation in the organism (see the example for the thermal window in Fig. 1.5 ; Pörtner, 2012). Thus, finding molecules related to the heat shock response and an antioxidant activity may indicate that individuals are in the acclimation phase, i.e., environmental stressors are within its pejus thresholds (low and high T_p interval, Fig 1.5).. Molecules related with anaerobic metabolism and metabolic depression may indicate acclimation in protection, i.e., stressors may pass out the critical thresholds of the species. Whereas a high accumulation of heat shock proteins and antioxidants may indicate acclimation in repair due to lethal thresholds.

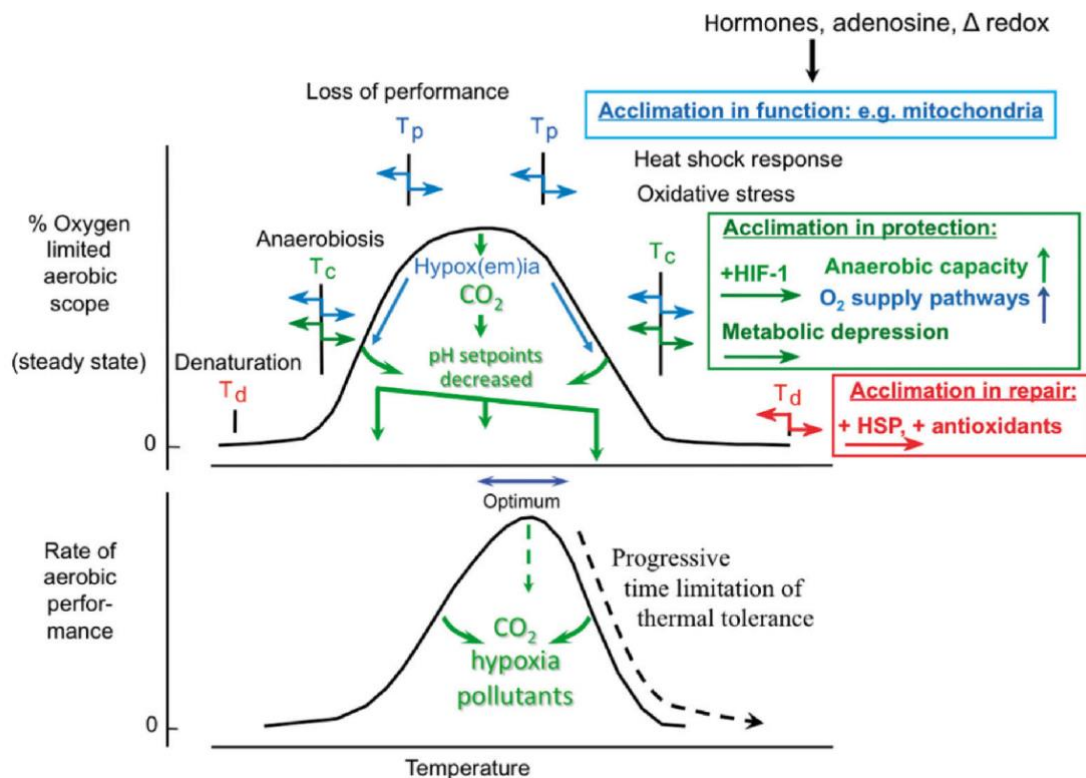


FIG. 1.5: Mechanisms involved in thermal thresholds and their interactions with hypoxia and acidification. T_p , T_c and T_d are the pejus, critical and lethal levels of temperature, respectively (from Pörtner, 2012).

The proteome (ie. all the proteins produced by the genome) are used to study the dynamic processes which depend on the physiological state of the organisms (Somero, 2012). Identifying the proteins involved in stress response is of major interest for understanding the underlying biological mechanisms (Artigaud, 2013). For example, heat stress should induce an alteration in the three-dimensional structure of proteins ; as a response, molecular chaperones may degrade or return denatured proteins to their original functional structure (Hartl, 1996 ; Wickner et al., 1999). Identifying molecular chaperones may indicate an active homeostasis and probably that the organism becomes thermally limited (between T_c and T_d , Fig. 1.5).

Initial physiological and biochemical adjustments under hypoxia depend on whether organisms are oxygen conformers or regulators (Fig. 1.4). When oxygen availability decreases, several marine invertebrates may increase water pumping/ventilation, blood perfusion rate, and cardiac activities (Herreid, 1980 ; Grieshaber et al., 1994). After a longer exposure, the marine invertebrates reduces its energy delivery and starts anaerobic metabolism to survive (Herreid, 1980 ; Wu, 2002). In response to severe hypoxia, the use of anaerobic glycolysis results in the accumulation of lactate or opines in the cytosol of marine organisms (Grieshaber et al., 1994). To produce energy anaerobically, instead of using the enzyme lactate dehydrogenase, mollusc use another pyruvate reductase called opine dehydrogenase (Grieshaber et al., 1994), the opine pathways being widespread in marine invertebrates (Harcet et al., 2013). In this biochemical pathway, pyruvate is condensed with an amino acid to form an imine acid (octopine, alanopine, strombine, tauropine, or nopaline), while regenerating one

mole of NADH + H⁺ (reduced form) into NAD⁺ (oxidized form ; Grieshaber et al., 1994). As these pathways do not accumulate lactate as final product (Harcet et al., 2013), there is no change in the pH and the enzymes are less disturbed (Zammit and Newsholme, 1976 ; Bowlus and Somero, 1979). In marine invertebrates exposed to hypoxia, octopine dehydrogenase (Odh) is more active than the lactate dehydrogenase (Ldh), allowing the synthesis of octopine from the condensation of pyruvate and arginine (Grieshaber et al., 1994). Octopine also has a role in supplying energy when tissues are anoxic after swimming : during scallop recovery, enhanced aerobic metabolism promotes octopine elimination and restoration of arginine phosphate as source of ATP (Livingstone, 1983).

Most invertebrates drastically reduce their metabolic rate during sustained severe environmental hypoxia. This capacity of metabolic depression in certain animal species is as important as the specific metabolic adaptations in order to withstand stressful environmental conditions (Hochachka, 1982). Metabolic depression, defined as a reduction in energy requirements, allows a metabolic regulator to be more tolerant to changes of suboptimal intercellular conditions (Grieshaber et al., 1994). Under metabolic depression metabolic reactions need not proceed at high velocities which allows reducing energy use, thus the accumulation and perturbation of the intracellular milieu due to anaerobic end products is limited (Grieshaber et al., 1994). The reduction of protein synthesis is a physiological compensation that allows reducing energy expenses through a decrease in general metabolism (Storey and Storey, 1990 ; Via et al., 1994 ; Hochachka, 1997). Since about 80% of the oxygen consumed by the mitochondria is coupled to ATP synthesis during aerobic respiration (Rolfe and Brown, 1997), the transition toward the hypometabolic state considerably reduces oxygen consumption.

In marine invertebrates, the ability to enter in a state of metabolic depression, characterized by maintaining a reduced but balanced equilibrium between ATP production and consumption, allows preventing a drop in cellular ATP concentration down to lethal levels (Grieshaber et al., 1994). Metabolic depression is the most protective and the most conserved adaptive mechanism within hypoxia-tolerant animals (Hochachka and Somero, 2002). If hypoxia lasts longer, ATP production becomes mitochondrial, by the “aspartate-succinate” pathway. The propionate produced from succinate is excreted in the external environment and does not accumulate in cells (Grieshaber et al., 1994, Fig. 1.6). Glycogen is the main form of glucose reserve in animals and the main source of energy for anaerobic metabolism. The large reserves of glycogen found in marine invertebrates, up to 35% of the dry mass (de Zwaan and Zandee, 1972), also contribute to the high tolerance of these animals to hypoxia. Under ocean acidification, acid-base balance is a crucial process, always coupled with osmoregulation due to the required uptake of appropriate counter ions (Evans, 1984). Metabolic depression and metabolic imbalance may arise under hypercapnia, depending on the degree of compensation in acid-base status. For example, a metabolic depression of up to 45% of control values is produced due to extreme acidosis (Pörtner et al., 2004). Among marine invertebrates, the system of membrane proteins is responsible for intracellular pH homeostasis during metabolic depression ; it may modulate the cost of intracellular pH regulation (Pörtner, 1996).

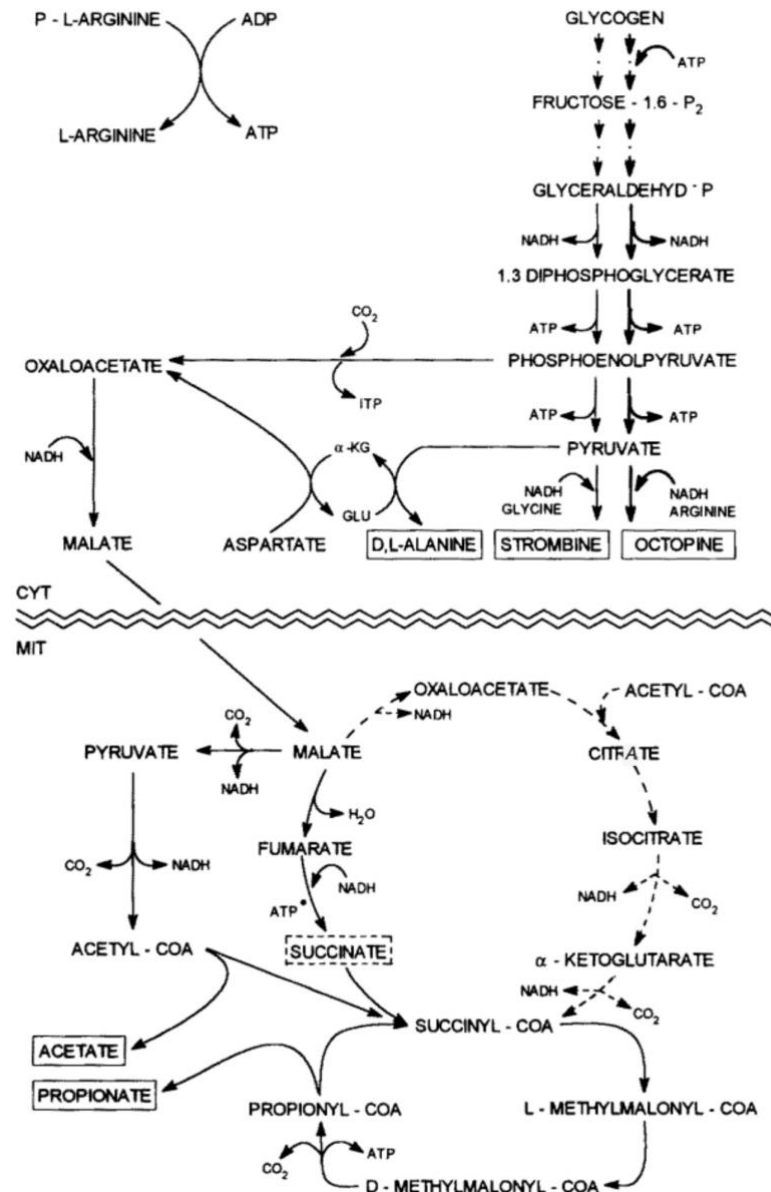


FIG. 1.6: Metabolic scheme of energy provision during anaerobiosis in the cytoplasmic (CYT) and mitochondrial (MIT) compartments of marine invertebrates (from Grieshaber et al., 1994).

The proteome is more related to the phenotype than to the genome (Biron et al., 2006). Adaptive phenotypic plasticity (ie. the expression of multiple phenotypes from one genome) may be crucial in allowing a species to withstand environmental change in the short temporal scales, before evolutionary adaptation is possible (Somero, 2012). In a productive but stressful environment such as HCS, the evaluation of the ecophysiological response of a benthic species such as *A. purpuratus* with high genetic variation, can give us indications of its potential vulnerability to climate change, and how this response at the individual-level can have consequences on the distribution and abundance of its population.

1.4 Research justification

Before 2013, most research about the Peruvian scallop, *A. purpuratus*, focused on field studies aiming at evaluating the scallops performance in terms of growth, reproduction and mortality, especially during the warm and cold phases of El Niño and with low (biweekly) frequencies (eg., Mendo Aguilar et al., 1989 ; Wolff and Mendo, 2000 ; Mendo et al., 2008). Then, description of the environment came with a higher frequency, e.g., environmental variations were assessed daily by measuring environmental conditions 3 times per day for periods of 1-3 months (e.g., Aguirre-Velarde, 2009 ; Cabrera Mendo, 2011). These works emphasized the necessity of better describing and understanding the environmental drivers in Peruvian coastal shallow waters (few meters). Between September 2012 and March 2013 (during spring and winter), a longer field monitoring with higher frequency (hourly) was initiated (Aguirre-Velarde et al., 2019b). This study showed that environmental conditions became critical during the summer : abrupt changes in temperature ($\pm 8^{\circ}\text{C}$) and a chronic and severe hypoxia (moderate and severe hypoxia correspond to 24 and 5% oxygen saturation, respectively ; Aguirre-Velarde et al., 2016) would have reduced the growth and reproduction, and induced scallop mortality. These conditions were worse on the seabed (e.g. moderate hypoxia was present during 47% of the survey time) than in the water column.

There are some physiological experiments carried out on the Peruvian scallop that focused on the response of the species under different types of hypoxia. Laboratory experiments of oxygen consumption under progressive hypoxia at two levels of temperature (16 and 23°C) highlighted the ability of the species to regulate its oxygen consumption in a wide range of temperature and a critical oxygen saturation of 24.4% (Aguirre-Velarde et al., 2016). Furthermore, under severe cyclic hypoxia (from 100 to 5% oxygen saturation), the scallops continued feeding, but at lower rates (Aguirre-Velarde et al., 2018). Bioenergetic models, based on Dynamic Energy Budget theory, suggested that other stressors than hypoxia may explain the observed reduction in scallop growth and reproduction (Aguirre-Velarde et al., 2019a). These studies highlighted the need to continue studying the effect of hypoxia on benthic organisms, but focusing on its interrelation with other environmental stressors. In order to do so, the high-frequency monitoring set up in Paracas Bay (Central-southern coast) in 2012 continued in the following years, while a second high-frequency monitoring was set up in the Sechura Bay (Northern coast). During these periods, scallop mass mortalities were recorded putting the fishermen and farmers at risk.

On the other hand, certain studies in bivalves suggested a size-dependent ability to cope with hypoxia, with responses which may vary depending on hypoxia intensity (Wang and Widdows, 1991 ; Shumway et al., 1983 ; Seto et al., 2004 ; Hicks and McMahon, 2005). Assessing the effect of size allows one to better understand the ability of the individuals to cope with environmental stressors throughout their life cycle. For example, ontogenetic variations such as early maturity (at lower body size) or early aging can affect the reproductive potential (due to reduced fertility and reduced spawning), and subsequently population recruitment and sustainability of the species.

1.5 Thesis objectives

In this context, this thesis aims to assess the ecophysiological responses of *A. purpuratus* to hypoxia and acidification, and how these responses vary with individual size. We replicate real or anticipated extreme local events along the Peruvian coast in our experimental conditions.

Chapter 1 provides an overview of the main points relevant to this thesis.

In Chapter 2, we analyze the consequences and potential risks posed by environmental stressors to scallop populations in two Peruvian bays. To achieve this, we conducted environmental surveys in Paracas and Sechura bays and performed a laboratory experiment to assess scallop survival under severe hypoxia conditions (1% oxygen saturation) at different sizes. The results of this study have been published in a scientific publication (Article 1, published).

Chapter 3 aims to better understand the effect of individual size by examining respiratory rates under progressive hypoxia and recovery processes following short exposure to severe hypoxia. We conducted a progressive hypoxia experiment, ranging from 100% to 5% oxygen saturation, to estimate the oxygen critical point for different scallop sizes. Additionally, we compared the oxygen debt generated after severe hypoxia (5% oxygen saturation) at varying sizes. The results and discussions are presented in the form of a scientific publication (Article 2, in preparation).

Chapter 4 compares the ecophysiological responses to combined hypoxia and acidification, identifying changes over time and potential effects on juvenile maturation. We assessed various ecophysiological responses, including mortality, reproduction, feeding, growth, calcification, and reproductive potential, to understand how medium-term exposure (51 days) to the two stressors affects scallop performance. We also investigated whether combining the stressors has a more detrimental effect than single treatments. These findings are presented in a scientific publication (Article 3, submitted).

Chapter 5 aims to gain a deeper understanding of the molecular mechanisms and differences between exposure to a single stressor and the combination of both stressors. This is achieved through a proteomic study conducted in the experiment described in Chapter 4 (Article 4, in preparation).

Finally, in Chapter 6, we summarize and discuss the results from the preceding chapters and present management options to ensure the self-sustainability of wild scallop populations in Peruvian bays within the context of ongoing and future environmental perturbations induced by global changes.

Chapter 2

Size-based survival of cultured *Argopecten purpuratus* (L, 1819) under severe hypoxia

Published in *Journal of the World Aquaculture Society*

(2021, DOI: 10.1111/jwas.12777)

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Abstract

During recent years, mass mortalities of cultured scallops became more frequent in Peruvian bays. One of the main drivers related to these mortalities was hypoxic events. This study was designed to understand the hypoxia phenomenon in these bays and to assess how the Peruvian scallop, *Argopecten purpuratus*, can cope with reduced oxygen levels in seawater based on individual size. First, we analyzed the relationships between dissolved oxygen saturation and other environmental parameters (the Coastal El Niño Index, temperature, salinity and pH) in bottom seawater, the frequency and intensity of hypoxic events in two main culture areas (Sechura and Paracas Bays), and their relationship with scallop mass mortalities. We found that at low-frequency data, the warm phase of ENSO and coastal El Niño events were closely related to mass mortalities ; whereas at high-frequency data, hypoxia (less than 24% DO saturation) is a common environmental stressor in Sechura and Paracas Bays. In the second part, we performed experiments with two size groups of *A. purpuratus* (31 and 80 mm shell height ; seed and commercial size, respectively) to assess their respective survival time under severe hypoxia (1% oxygen saturation) at 16°C and a salinity of 35. Our results suggest that small *A. purpuratus* better cope with severe hypoxia (L_{t50} : 3.9 d) than larger ones (L_{t50} : 2.5 d). Besides, a higher condition index increased the survival along the two size groups. Finally, the results of the field records and the experimental data are used to suggest ways to improve scallop cultivation along the Peruvian coast.

Keywords : *Argopecten purpuratus*, L_{t50} , mass mortality, severe hypoxia, size effect.

2.1 Introduction

The Peruvian scallop, *Argopecten purpuratus* (Lamarck, 1819), is a significant resource in Peru and Chile and one of the few marine species cultured in Peru. Its production increased rapidly in the early 2000s due to fast growth (from 7 to 18 months to reach commercial size, depending on site and culture system) (Mendo et al., 2008, 2016) and lower production costs compared to Chile (Kluger et al., 2019b). In Peru, scallop culture activities support scallop farmer cooperatives and generate incomes to ~20,000 additional workers whose activities rely on scallop processing chains and associated businesses (Kluger et al., 2019a). Nevertheless, mass mortality events since 2012 have led to colossal scallop production drops (e.g., from 52,213 tonnes in 2011 to 24,782 tonnes in 2012, and from 67,694 tonnes in 2013 down to 11,927 tonnes in 2017 ; source : <http://www.fao.org/fishery/>) and have severely affected the artisanal farmer cooperatives from Sechura and Paracas Bays (two of the main scallop culture areas in Peru). In Sechura Bay (the most impacted area), the economic losses due to mass mortalities in 2017 reached close to \$1 million US dollars (source : <http://direpro.regionpiura.gob.pe/>).

Since 2012, eight mass mortality events have been recorded in Sechura Bay (2012, 2015, 2017, and 2019) and Paracas Bay (2012, 2013, 2015, and 2017). Although information about environmental conditions preceding these events is scarce, mortalities were commonly linked to increases in temperature and bottom oxygen-depleted processes (eutrophication, red tides, milky-turquoise waters) Aguirre-Velarde et al. (2016) ; Aguirre-Velarde et al. (2019b) ; Kluger et al. (2019a). Temperature rising during these events was related to the summer heat, to the warm phases of the El Niño Southern Oscillation IMARPE (2015), and to the Coastal El Niño Index ENFEN (2012) ; Takahashi et al. (2017). Low dissolved oxygen levels (hypoxia) in seawater may be human-induced but it is also a natural process in some marine ecosystems. The Peruvian Humboldt upwelling system is associated with a strong oxygen minimum zone (OMZ ; permanent hypoxic areas in the deep ocean ; (Graco et al., 2017 ; Altieri Diaz, 2019). Depending on years, sporadically advected OMZ water or high productivity associated with the coastal upwelling can lead to hypoxia in shallow coastal waters (Chavez et al., 2008 ; Levin et al., 2009). Both abrupt temperature increases and deoxygenation in seawater are magnified by climate change, resulting in increasing stress for marine organisms (Bopp et al., 2013 ; Gunderson et al., 2016).

Monitoring environmental dynamics is crucial because going over thresholds may induce acute or chronic stress in living organisms. The response to stressful conditions depends on the intensity and relative timing of single and combined environmental stressors. The consequences of multi-stressor exposure are commonly more negative than expected based on responses to single stressor (Gunderson et al., 2016). The upper lethal temperature generating 50% of mortality in *A. purpuratus* is $29 \pm 3^{\circ}\text{C}$ Urban (1994), a value never recorded in Peruvian bottom waters even during very intense El Niño events Tarazona et al. (1988) ; Arntz et al. (1991) ; Mendo and Wolff (2003) ; Kluger et al. (2019a). This species seems well-adapted to high temperatures since it exhibited enhanced growth and reproduction during the warm ENSO events of 1983 and 1998 (Arntz et al., 1991 ; Wolff, 1987 ; Yamashiro et al., 2002). Lethal oxygen levels are unknown for *A. purpuratus*, but Aguirre-Velarde et al. (2016) classified this

species as a good oxy-regulator under broad oxygen levels at both 16°C and 25°C : the respiration rate of the species starts to decrease below dissolved oxygen saturation (DO_{sat}) of 24.4% (2.02 ppm at 25°C), until ca. 22% of its normoxic value at 5% DO_{sat} (0.44 ppm at 25°C). Another study showed that *A. purpuratus* kept feeding and growing after 21 days under cyclic hypoxia at 5% DO_{sat} (Aguirre-Velarde et al., 2018). Nevertheless, more than 50% of bottom-cultured scallops died after chronic and severe hypoxia in Paracas Bay, showing slight size-related mortality Aguirre-Velarde et al. (2019b).

Some bivalves show size-dependent survival under hypoxia (Hicks and McMahon, 2005 ; Seto et al., 2004 ; Shumway et al., 1983 ; Wang and Widdows, 1991), which can vary according to oxygen concentration (Hicks and McMahon, 2005) and temperature (Shumway et al., 1983). Robb and Abrahams (2003) hypothesized that increasing body size reduces the ability to cope with hypoxia. Increasing size is related to decreasing surface-to-volume ratio and increasing oxygen diffusion distance, while higher body weight in larger individuals means higher energetic demand (Robb and Abrahams, 2003 ; Wang and Widdows, 1991). Hence, oxygen limitation and anaerobic metabolism would start earlier in larger individuals than in smaller ones. Under prolonged hypoxia or anoxia however, bivalves consume their body energy reserves (Grieshaber et al., 1988) and the larger individuals have an advantage compared to the smaller ones due to higher reserves (Nilsson and Östlund-Nilsson, 2008). However, anaerobic pathways produce harmful metabolites (de Zwaan and Wijsman, 1976 ; Hochachka, 1997 ; Grieshaber et al., 1988), which may affect the organisms if the hypoxic stress is prolonged.

The degree of individual tolerance to environmental stressors (focusing on severe hypoxia) and the relationship between size and survival are required for better understanding of scallop mortality events and better management of scallop culture in Peru. The present work aimed to analyze the consequences and potential risks of hypoxic conditions for scallop cultivation in two Peruvian bays by coupling environmental surveys with a laboratory experiment on scallop survival. Field surveys were carried out in the cultivation areas of Sechura Bay (Northern coast) and Paracas Bay (Central-southern coast) between the years 2013 and 2019. The laboratory experiment focused on survival time under severe hypoxia according to individual size. The two size groups studied, large (80 ± 3.1 mm SD shell height) and small (31 ± 3.6 mm SD), were representative of commercial size (for consumption) and bottom-collected seed size, respectively. Finally, we used the relevant information on the scallop mortality events, the degree of individual tolerance to environmental stressors, and the relationship between size and survival to formulate some suggestions for better management of scallop aquaculture in these bays, which may contribute to the sustainability of this activity.

2.2 Material and methods

2.2.1 Field analysis of environmental conditions and mortality in the two main scallop production areas

Study sites

Two main scallop growing areas in Peru were investigated : Sechura (5°40' S, 80°54' W ; Piura region) and Paracas (13°50' S, 76°16' W ; Ica region ; Figure

2.1) Bays. *A. purpuratus* is the unique species being cultured in both bays. The average relative production in the Piura and Ica regions was 47.2 and 2.1% of the total Peruvian scallop production between the years 2008 and 2018, respectively (<http://ogeiee.produce.gob.pe/index.php/>).

Sechura Bay, located in the north of Peru, is in a transitional zone between the Humboldt current (cold waters) and the Equatorial current (warm waters), meanwhile Paracas Bay, located in the central-southern coast, is mainly influenced by the Humboldt current. The total surface area of Sechura Bay and Paracas Bay is 1,120 and 36 km², respectively. Mean water depth is about 15 m in Sechura (max. depth : 50 m) versus 5 m in Paracas (max. depth : 14 m). The same sediment type can be found in both bays, that is a mixture of gravel, sand, and mud.

Sechura Bay receives little freshwater discharge from the Piura River through the Virrilá estuary overall, but this discharge can strongly increase during intense ENSO events (11.47 million m³ in 1983 ; Salas et al., 2014), due to high precipitation (total rainy season rainfall in Piura up to 2.14 m in 1982–1983 ; Takahashi, 2004). In contrast, Paracas Bay does not receive freshwater discharges, the nearest inlet (Pisco seasonal river) is located 14 km further north. Rarely the Pisco river plume may enter the bay when strong winds switch to the southward direction (Merma, personal communication). Extreme events such as algal blooms and sulfur plumes are often observed in both bays (Aguirre-Velarde et al., 2019b ; Chauca Vela, 2018 ; Gonzales et al., 2012 ; IMARPE-Pisco, 2012 ; IMARPE, 2015, 2019 ; Morales, 2017 ; Sánchez et al., 2015).

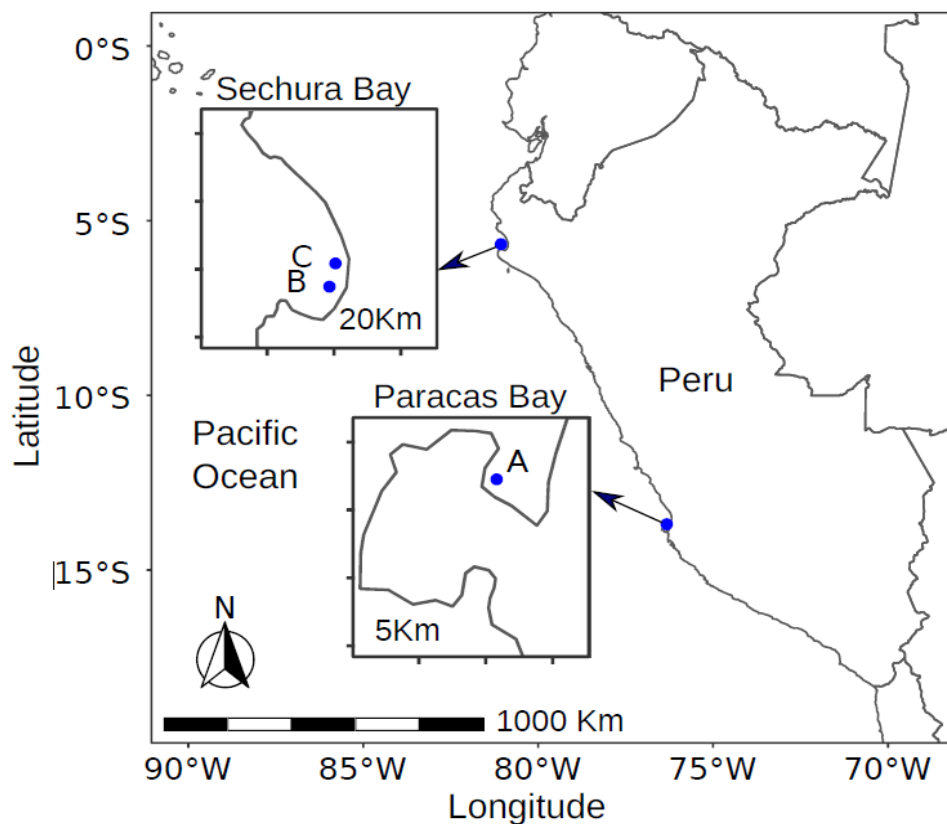


FIG. 2.1: Location of the two *Argopecten purpuratus* culture areas studied along the Peruvian coast. Site names : C : Constante, B : Barrancos, and A : Atenas. See Table 2.1 for site geographical coordinates

Descriptors of environmental conditions

Prior to the analysis of the environmental stressors in the studied bays, we performed an environment characterization using the ICEN and four local descriptors (temperature, DO, salinity, and pH). The ICEN is defined as the three-month running mean of sea surface temperature (SST) anomalies in the “Niño 1 + 2” region (ENFEN Comisión, 2012 ; Takahashi et al., 2014). The ICEN characterizes the coastal conditions better than the more widely used Oceanic Niño Index (ONI : <http://www.cpc.ncep.noaa.gov/>) ; it is also sensitive to regional events such as the coastal event that occurred in 2017–2018 (Garreaud, 2018). ICEN categorizes as coastal El Niño events (CEN) events during which SST anomalies of $+0.4^{\circ}\text{C}$ occur for three consecutive months, and as coastal La Niña events (CLN) events during which SST anomalies of -1.0°C occur for three consecutive months.

Seawater environmental conditions were defined compiling data from various sources : (1) data records from The National Fisheries Health Agency of Peru (SANIPES : <http://179.43.86.24/web-oceano/>), (2) data records from two companies (PRISCO scallop farming and ERM environmental consultant), and (3) data loggers from this study. Biweekly records of temperature, dissolved oxygen saturation, salinity, and pH were obtained from SANIPES in both sites between 2013 and 2019. From this data set, we get records from near the bottom (1 m above the bottom), and at 6 m and 14 m above the bottom, depending on the station. Daily records of temperature, DO_{sat} , and salinity were obtained from PRISCO in Sechura Bay, while daily records of temperature, DO, and pH in Paracas Bay were obtained from ERM. Finally, between 2012 and 2019, autonomous data-loggers of temperature (HOBO U22-001 ; Onset Corp., Bourne, MA), DO_{sat} (DO-1060 ; RBR Ltd., Ottawa, Canada), and the combined temperature/DO loggers TDO2050 (RBR Ltd., Ottawa, Canada) and MiniDOT (PME Inc., Vista, CA) were tied on a metal frame placed 0.2 m above the bottom by SCUBA divers in both bays (details in Table 2.1).

Review of scallop mortality events in Paracas and Sechura Bays

Mortality events of *A. purpuratus* were documented from 2012 to 2019 in Paracas and Sechura Bays by using the statistics of the Peruvian Ministry of Production (PRODUCE, see www.produce.gob.pe), reports from the IMARPE and sea farmers, and peer-reviewed literature (Aguirre-Velarde et al., 2019b). Details of the records are given in Table 2.2.

TAB. 2.1: Environmental parameters recorded near bottom in Paracas and Sechura Bays between 2012 and 2019.

Period of data recording	Parameter	Station	Depth	Data source	Recording frequency
Sechura Bay					
January 03, 2012 - November 09, 2016	Temp, DO _{sat}	C	12 m	This study (a)	hourly
June 11, 2014 - March 16, 2015	Temp, DO _{sat}	B*	12 m	This study (a)	hourly
March 23, 2017 - May 24, 2017	Temp, DO _{sat}	C	12 m	This study (a)	hourly
January 23, 2018 - March 01, 2019	Temp, DO _{sat}	C	12 m	This study (b)	hourly
January 23, 2012 - March 06, 2017	Temp, DO, Sal	C	12 m	PRISCO data (d)	daily
January 02, 2013 - September 02, 2017	Temp, DO _{sat} , Sal, pH	C	12 m	Sanipes data (d)	biweekly
Paracas Bay					
August 28, 2012 - March 10, 2013	Temp, DO _{sat}	A	5 m	This study (c)	hourly
February 20, 2015 - March 21, 2015	Temp, DO _{sat}	A	3 m	This study (b)	hourly
March 19, 2016 - October 14, 2017	Temp, DO _{sat}	A	5 m	This study (b)	hourly
January 02, 2013 - September 02, 2017	Temp, DO _{sat} , Sal, pH	A	5 m	Sanipes data (d)	biweekly
January 01, 2012 - October 10, 2015	Temp, DO, pH	A	5 m	ERM data (d)	daily

(a) Dataloggers HOBO U22-001 and RBR DO-1060.

(b) Datalogger MiniDOT.

(c) Datalogger RBR TDO.

(d) Datalogger Low frequency data from Prisco, Sanipes and ERM were used to complete the high-frequency records and characterize the monthly relationships between the four environmental parameters analyzed in this study.

*The Barrancos data were used to replace missing records in Constante during or surrounding a mass mortality event. Barrancos and Constante stations are close and exhibit similar temperature and oxygen conditions.

Abbreviations: DO, dissolved oxygen concentration (mg/L); DO_{sat}, dissolved oxygen saturation (%); Sal: salinity, and pH; Temp: temperature (°C); Station refers to the location of the probes: C: Constante (5°40'33.3" S, 80°54'1.6" W) and B: Barrancos (5°47'33.6" S, 80°55'44.8" W) in Sechura Bay; A, Atenas (13°50'35.8" S, 76°15'59.5" W) in Paracas Bay.

TAB. 2.2: Mass mortality events recorded in scallop (*Argopecten purpuratus*) bottom cultures between the years 2012 and 2019 in Sechura and Paracas Bays.

Periods of data recording	Mortality	Mortality causes*	ICEN	Data sources
Sechura Bay				
January 06, 2012 - February 06, 2012	90 %	A, RT, TI, MTW	-0.4 (N)	ENFEN (2012); Gonzales et al. (2012)
March 02, 2015 - March 14, 2015	ca. 100 %	RT, A	0.18 (N)	PRISCO, pers. com.
January 17, 2017 - February 08, 2017	90 %	A, RW, MTW4	0.6 - 0.93 (WEN-MEN)	Sanchez et al. (2015); Kluger et al. (2018)
March 04, 2019 - March 12, 2019	ca. 100 %	RT, A, RW, RT	0.25 (N)	IMARPE (2019)
Paracas Bay				
April 02, 2012 - April 04, 2012	ca. 100 %	A, RW, RT	0.66 (WEN)	Morales (2017)
February 28, 2013 - March 10, 2013	ca. 100 %	A, MTW	-0.79 (N)	Aguirre-Velarde et al. (2019b)
March 03, 2015 - March 19, 2015	ca. 100 %	TI, A, MTW, RW	0.18 (N)	Morales (2017)
March 09, 2017 - March 17, 2017	70-90 %	RT, A, MTW	0.93 (MEN)	Cook (2017)

*Mortality causes suggested by the authors.

Abbreviations: A, anoxia; ICEN, Corresponding ENSO phases; MEN, Moderate El Niño; MTW, milky-turquoise waters and/ or sulfur plumes records; N, Neutral phase; RT, red tides; RW, freshwater inputs from the river; TI, temperature increases; WEN, weak El Niño.

Data analyses

To explore the spatio-temporal relationship between the bottom-water (bw) conditions in Sechura and Paracas Bays, the monthly averages of temperature, DO_{sat}, salinity, and pH were compared by using principal component analysis (PCA). All variables were scaled to be zero-centered before the PCA. Significant grouping in abiotic conditions according to the ENSO phases and seasons was tested by using the Wilks test (significance level of $\alpha = .05$). The ENSO phases (according to the ICEN index, ENFEN Comisión, 2012 ; Takahashi et al., 2014) used were neutral phase (N), weak El Niño (WEN), moderate El Niño (MEN), strong El Niño (SEN), weak La Niña (WLN), moderate La Niña (MLN), and strong La Niña (SLN). All statistical analyses were performed using the R software (version 3.4.4 ; R Core Team, 2018) ; PCA was performed using the “FactoMiner” R package (Husson et al., 2010).

The high frequency (hourly) data (temperature, DO_{sat}) available during the 21 days preceding the mass mortality peaks in the field were compared to assess the local environmental conditions during mortality events in both bays (see Table 2.2). For this analysis, we used temperature and oxygen saturation physiological thresholds for *A. purpuratus* from Peru reported by Aguirre-Velarde et al. (2016) and Urban (1994). To evaluate a possible stress produced by sudden

temperature increases in bottom seawater, the hourly records of bw temperature during the mortality events were compared with the L_t value after 24 h (temperature at which 50% of the experimental population was dead after 24 h) estimated by Urban (1994) for scallops originating from the Bay of Independencia in Peru (29°C ; 95% CI : 26–32°C).

The hypoxic stress was studied from two thresholds of oxygen saturation, the critical DO_{sat} of 24% and the 5% DO_{sat} , in order to assess the starting of oxy-conformity and metabolic depression for the Peruvian scallop (Aguirre-Velarde et al., 2016, ; more details in the introduction). By using the temperature and oxygen thresholds mentioned above, moderate heat and hypoxia were defined by temperature below 23°C and 5–24% DO_{sat} , respectively. Severe hypoxia corresponded to DO_{sat} below 5%. Any common stress conditions previous to mass mortality events were assessed by analyzing the relative proportions of moderate and severe hypoxia against normoxia in the 21 days before mass mortality events (Pearson's chi-squared test). For all statistical analyses, a significance level of $\alpha = .05$ was used. Additionally, to have detailed descriptors of hypoxia along the year, we calculated the percentage of hours per month with hypoxic conditions (P, %) and the duration of hypoxic events (D, consecutive hours under hypoxia), using the thresholds mentioned previously, for all the period analyzed (January 2012 to December 2019).

2.2.2 Survival time under severe hypoxia experiment

Biological material and acclimation procedure

A total of 80 small (mean 30.9 ± 3.1 mm SD, shell height) and 80 large (mean 79.7 ± 3.6 mm SD, shell height) scallops were collected in October 2017 in Paracas Bay by Scuba diving. Individuals were transported in a cool box to the Aquatic Ecophysiology Laboratory at the IMARPE (Callao, Peru). Once in the laboratory, scallops were distributed into 80-L tanks for acclimation (20 large-sized and 40 small-sized scallops per tank, respectively). Tanks were supplied with 1- μ m filtered seawater at a constant flow of 6 L/hr. The seawater temperature and salinity were maintained at $16.1 \pm 0.2^\circ\text{C}$ and 34.9 ± 0.1 , respectively. During acclimation, scallops were fed a mix of cultured microalgae composed by *Isochrysis galbana* (IMP-BG-010) and *Chaetoceros calcitrans* (in a 1:1 cell proportion) three times a day to maintain an average concentration of 9.2×10^6 cells/L in the tanks. This cell concentration was shown to allow *A. purpuratus* growth after 21 days in laboratory conditions (Aguirre-Velarde et al., 2018). Due to technical issues, individuals were acclimated for 6 to 9 weeks before starting the experiments. This long acclimation time did not seem to affect the condition index of both size groups prior to the experiments. Food supply was stopped 1 day before starting the experiments.

Experimental conditions

Four experiments (one control and three replicate hypoxic conditions) were performed between December 2017 and January 2018. For each replicate, 40 scallops (20 small-sized and 20 large-sized individuals) were placed into a 40-L aquarium without food and exposed to severe hypoxia (1% DO saturation) until 100% mortality was reached. Experimental aquaria were continuously supplied with 1- μ m filtered and thermoregulated (16°C) seawater (1.8 L/hr). DO_{sat} in the aquarium of the severe hypoxia treatment was maintained to 1% (close to 0.4 mg/L at 16°C) using a computer-controlled gaseous nitrogen injection system

(described in Artigaud et al., 2014) interfaced with an FDO 925-3 dissolved oxygen sensor (WTW, USA) allowing to continuously monitor temperature and DO_{sat}. Gas exchanges with the ambient air were limited by placing a floating polystyrene sheet on the water surface. The normoxic condition (c.a. 90% DO_{sat}) in the control was reached by bubbling air into the tank. The control experiment was performed using the same size groups and numbers of individuals as the hypoxic treatments.

Measurements

Mortality was assessed twice a day by the failure of tissue constriction after the valves of gaping animals were pressed. Shell height (H, mm) of each dead scallop was measured to the nearest 0.1 mm using a Vernier caliper ; then, soft tissues (muscle, gonad, gills, and mantle tissues) were dissected, dried (96 hr at 60°C), and weighed to the nearest 0.1 µg (DW, g). The Fulton's condition index (CI, g/cm³) was calculated as $CI = 1,000 (\text{soft tissue DW})/(\text{shell height})^3$.

Data analyses

The survival span for each size group (small-sized and large-sized) was calculated as the number of days from the beginning of the exposition to death. These data were used in the construction of the Kaplan–Meier survival curves, allowing to estimate the Lt₅₀ and Lt₉₀ (time at which 50 and 90% of the population die, respectively) and the confidence limits for each size group (Kaplan Meier, 1958). The log-rank test was used to test the possible differences in survival between the size groups (Mantel, 1966). The possible effect of the condition index or experimental time on survival time was tested using Cox proportional hazard models ($p < .05$ from the Wald test statistics ; Therneau Grambsch, 2013). All statistical analyses (significance level of $\alpha = .05$) were performed using the “survival” and “survminer” packages (Therneau & Grambsch, 2013) of the R software (version 3.4.4 ; R Core Team, 2018).

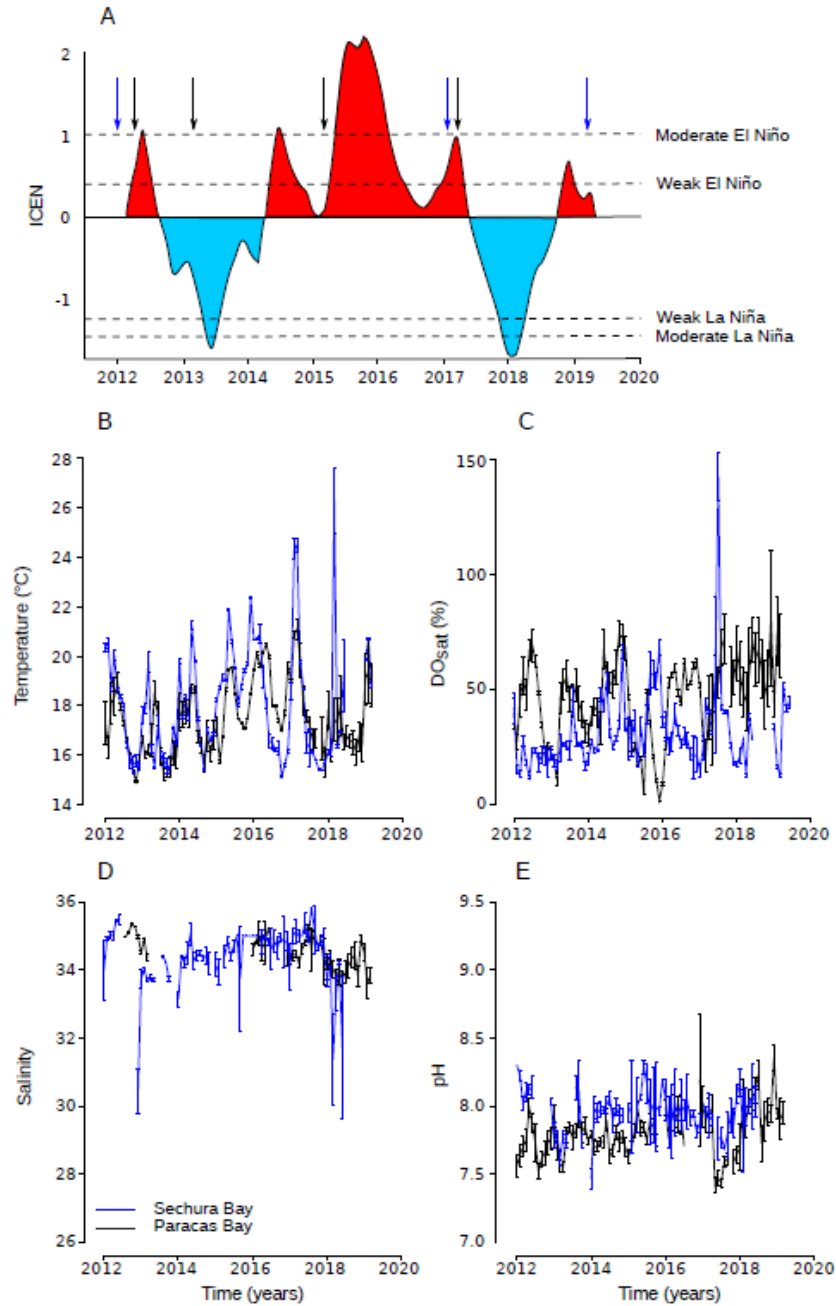


FIG. 2.2: Environmental bottom seawater conditions available in Sechura and Paracas Bays between the years 2012 and 2019. (a) Coastal El Niño index (ICEN), (b) Monthly averages of bottom-water temperature, (c) dissolved oxygen saturation, (d) salinity, and (e) pH. Blue and black lines indicate the mean values in Sechura and Paracas Bays, respectively. Error bars represent 95% confidence intervals. Blue and black arrows indicate the occurrence of scallop mass mortalities in Sechura and Paracas (details in Table 2.2). Environmental variables have a greater variability in Sechura than in Paracas. Monthly temperature and salinity averages in both bays exhibited similar seasonal patterns (Pearson correlation test, $p < .01$; $R = .595$ and $.429$, respectively).

2.3 Results

2.3.1 Abiotic conditions in Sechura and Paracas Bays

The ICEN index, monthly averages of temperature, DO saturation, salinity, and pH were highly variable between the years 2012 and 2019 in Sechura and Paracas Bays (Figure 2.2). Sechura Bay showed the highest variability in the average of monthly temperatures ($18.0 \pm 2.1^\circ\text{C}$ SD ; maximum and minimum values of 31.6 and 14.5, respectively). Paracas showed a bit lower variability than Sechura ($18.2 \pm 1.9^\circ\text{C}$ SD ; maximum and minimum values of 25.7 and 13.8°C, respectively). The rise in temperature was mainly related to the summer austral season. Monthly temperature averages in both bays exhibited similar seasonal patterns (Pearson correlation test, $p < .01$; $R = .595$) along the years with slight differences in the extreme values (Figure 2.2b). The monthly percentage of DO_{sat} was $36.3 (\pm 1.4 \text{ SD})$ and $38.1\% (\pm 1.9 \text{ SD})$ in Sechura and Paracas Bays, respectively. Though data sets available for pH and salinity were scarce, Sechura Bay also showed more variability in the averages of monthly salinity and pH. Average monthly salinity was $34.5 (\pm 0.6 \text{ SD})$ in Sechura versus $34.7 (\pm 0.2 \text{ SD})$ in Paracas. Mean pH was $7.9 (\pm 0.2 \text{ SD})$ and $7.8 (\pm 0.1 \text{ SD})$ in Sechura and Paracas, respectively.

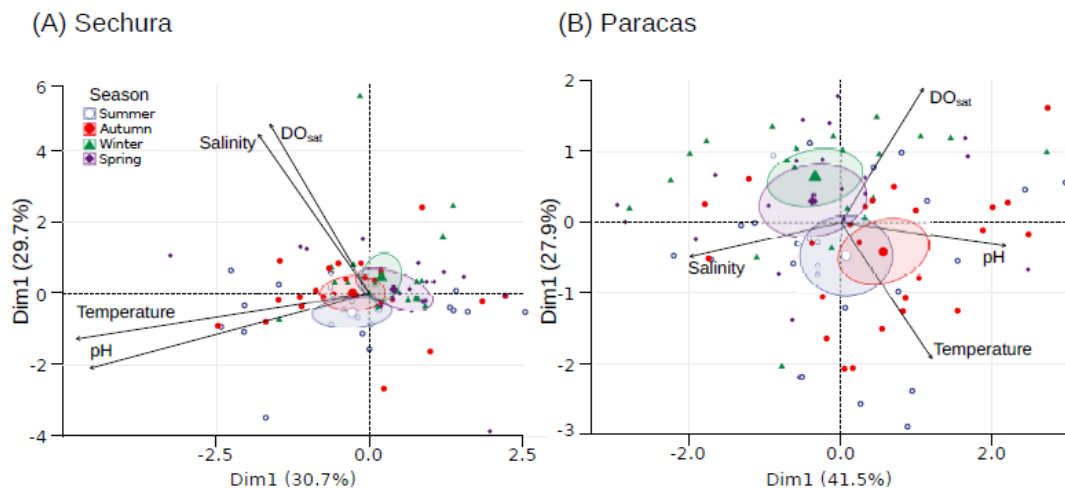


FIG. 2.3: Principal component analysis of the monthly average conditions in bottom seawater at Sechura and Paracas. Biplots represent the two first principal components with 60.39 and 69.38% of the total variability explained in Sechura and Paracas, respectively. DO_{sat}: dissolved oxygen saturation ; Sal : salinity ; Temp : temperature. There were significant differences ($p < .0001$) between the ENSO phases and seasons at both sites. Ellipses indicate 95% confidence limits of the seasonal average bottom-water-condition groupings.

PCA analysis between the ENSO phases and monthly averages of environmental parameters in both bays showed that the first and second principal components contributed 60.4 and 69.4% to the global variability at Sechura and Paracas, respectively. The relationships between the abiotic variables (temperature, oxygen, salinity and pH) differed between both bays. In Sechura, clear positive correlations were found between salinity and DO_{sat} on one hand, and between temperature and pH on another hand. In Paracas, there was a strong negative correlation between temperature and DO_{sat} on one hand, and

between salinity and pH on another hand (Figure 2.3). Besides, the abiotic conditions were significantly different depending on the ENSO phases (Wilks' $\lambda = .50$, $p < .0001$ and Wilks' $\lambda = .44$, $p < .00001$, for Sechura and Paracas, respectively) and seasons (Wilks' $\lambda = .57$, $p < .00001$ and Wilks' $\lambda = .56$, $p < .00001$, respectively).

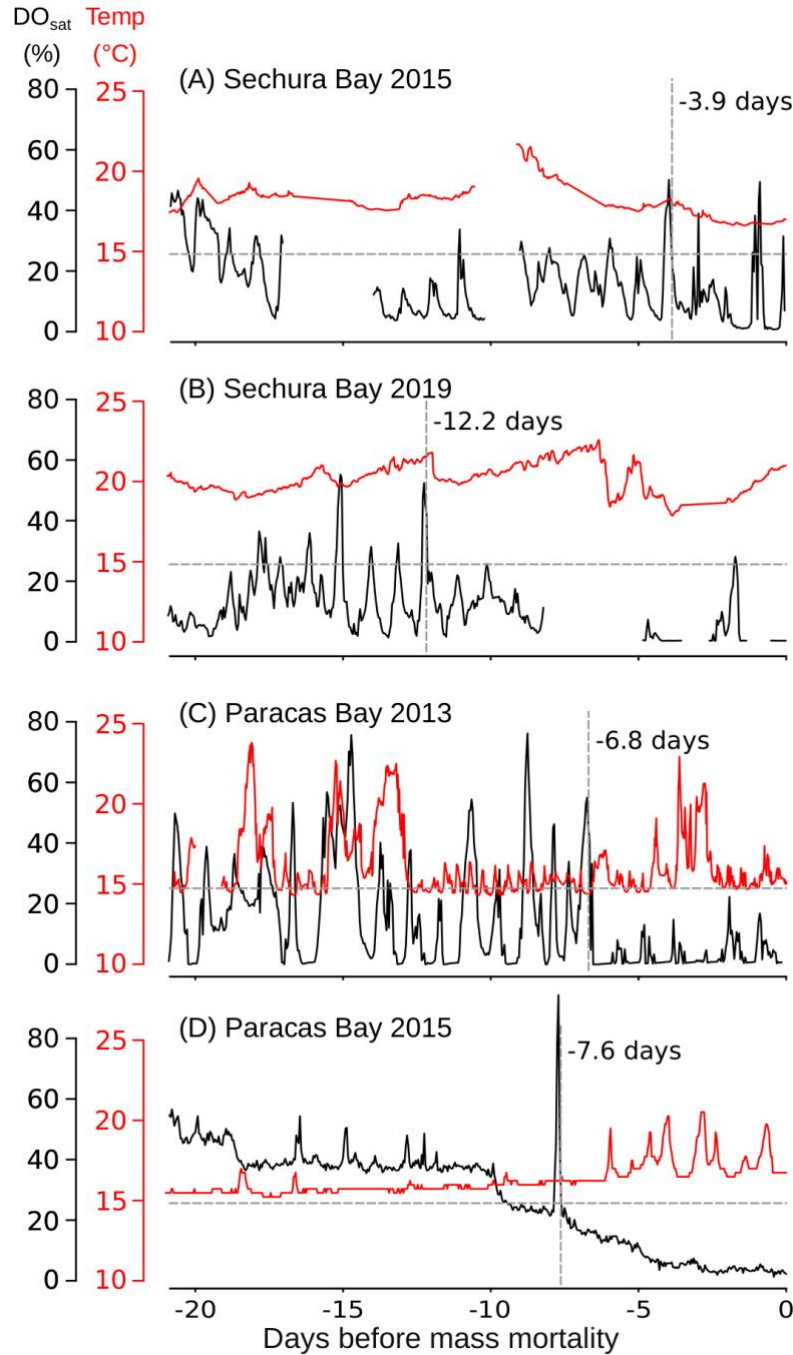


FIG. 2.4: Hourly temperature (red lines) and dissolved oxygen saturation (black lines) in the 21 days before scallop mass mortalities recorded in Sechura and Paracas Bays : (a) March 14, 2015, (b) March 12, 2019, (c) March 10, 2013, (d) March 19, 2015. Vertical dashed lines : time from which DO saturation is below 24% for more than 90% percent of the time. Horizontal dashed lines : 24% DO_{sat}.

2.3.2 Temperature and hypoxic events versus scallop mass mortalities

Temperature did not seem to be a triggering factor for scallop mass mortality events which occurred in the two bays during the study period. There was no common heatwave in the 21 days before the mass mortalities recorded in Sechura and Paracas. Bw temperature was stable and close to 20°C in Sechura Bay during the 3 weeks preceding the mortality events of 2015 and 2019 (Figure 2.4). In Paracas, bottom-water temperature was lower (ca. 16°C) than in Sechura before the mortality events of 2013 and 2015 ; the higher value was observed in 2017 ($T^{\circ} > 20^{\circ}\text{C}$ 3 weeks before the mass mortality), but the lack of data afterward precludes to draw any conclusion.

By contrast, hypoxic conditions were present in all the periods preceding the mortalities. The former could last for more than 2 days, without more than three consecutive hours above the moderate hypoxia threshold (24% DO_{sat}). Hypoxia was present in both bays at both levels of intensity (moderate - 24% DO_{sat} and severe - 5% DO_{sat}), but the relative proportions of each hypoxic condition were significantly different between years (chi-square = 155.5, df = 8, $p < .0001$). Hypoxia occurs not only during mass mortality events, but it is frequent in austral summer (December to March) and early autumn (April and May, Table 2.3). The extent of bw hypoxic conditions along the year was higher in Paracas Bay (only the months of July and August were free from hypoxia), compared with Sechura Bay (6 months, from May to October, free of hypoxia ; Table 2.3). In Sechura, the longest episode of severe hypoxia occurred in April during the study period (58 hr, that is, 2.4 days), while the longest moderate hypoxia event happened in January (176 hr, that is, 7.3 days). In Paracas, the longest severe (95 hr, that is, 4.0 days) and moderate (312 hr, that is, 13 days) hypoxia episodes occurred in December (Figure 2.5). Whatever the bay and year, however, mass scallop mortalities were generally recorded in March (Table 2.2), which questions the multi-stressor combination generating such collapses. Last, the highest duration below or up to 1% DO_{sat} was 13 hr in both bays.

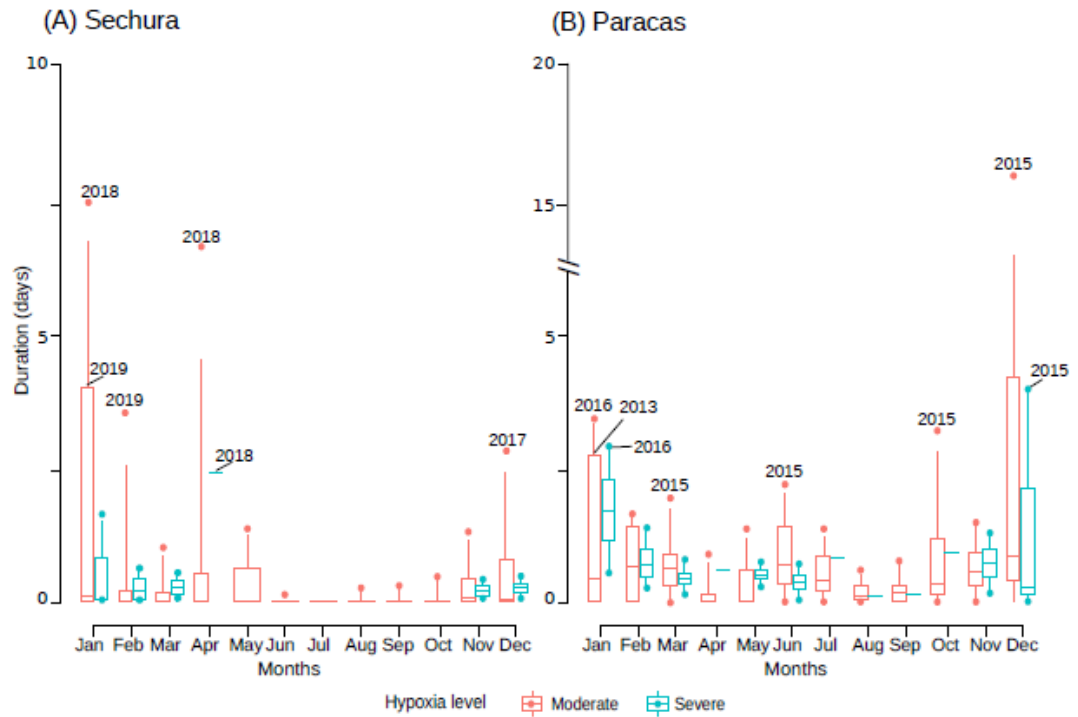


FIG. 2.5: Box-and-whisker plots of the longest duration of hypoxic events (in days, calculated from continuous hours of hypoxia) recorded by month/year in Sechura and Paracas Bays between 2012 and 2019. Horizontal lines within boxes are medians, the lower and the upper limits of the boxes are the 25th and 75th percentiles, respectively, and the whiskers extend to the 5th and 95th percentiles, respectively. Outliers of the longest monthly durations are indicated by points and years of occurrence. The duration of the hypoxic events are detailed in Table 2.3.

TAB. 2.3: Monthly characterization of hypoxic events in bottom waters in Sechura and Paracas Bays between January 2012 and December 2019. Note: F is the frequency is the number of interrupted events of severe (DO_{sat} below 5%) and moderate hypoxia (DO_{sat} below 24%). P (%) is the percentage of hours per day with DO_{sat} under severe or moderate hypoxia; duration (hours) represents the average consecutive hours (mean \pm SD) per month under severe or moderate hypoxia. Max is the maximum number of consecutive hours under the hypoxic stress conditions aforementioned. NA: SD-values not available (only one record of hypoxia in the month).

DO_{sat}	Month	Sechura Bay				Paracas Bay			
		Frequenc y	P (%)	Duration (hr)	Max (hr)	Frequenc y	P (%)	Duration (hr)	Max (hr)
5%	February, 2012	7	0.7	1.0 ± 0.0	1				
	March, 2012	3	0.3	1.0 ± 0.0	1				
	November, 2012					27	2.8	2.3 ± 1.1	6
	December, 2012					22	2.5	2.6 ± 2.3	7
	January, 2013					45	8.2	4.1 ± 3.7	14
	February, 2013					60	9.9	3.8 ± 3.5	17
	March, 2013					52	9.2	2.8 ± 2.2	10
	March, 2015					29	8.6	4.7 ± 3.6	12
	April, 2015					79	18.4	3.6 ± 3.3	15
	May, 2015					89	18.8	3.4 ± 3.2	17
	June, 2015					73	13.1	2.7 ± 3.0	16
	July, 2015					172	31.7	2.9 ± 2.9	20
	August, 2015					4	0.4	1.5 ± 1.0	3
	September, 2015					11	0.9	1.9 ± 1.2	4
	October, 2015					46	11.5	5.7 ± 5.4	23
	November, 2015					114	20.3	4.0 ± 4.5	30
	December, 2015					28	30.6	25.1 ± 22.1	95
	January, 2016					60	19.1	7.2 ± 12.6	69
	February, 2016					48	12.7	6.1 ± 6.1	32
	March, 2016					30	8.5	4.4 ± 4.1	18
	May, 2016					9	1.3	2.2 ± 2.6	9
	June, 2016					2	0.3	2.5 ± 0.7	3
	November, 2016	9	1.1	1.0 ± 0.0	1				
	December, 2016	13	1.9	1.2 ± 0.6	3	2	0.1	1.5 ± 0.7	2
	January, 2017	1	0.1	$1.0 \pm NA$	1				
	February, 2017	7	0.8	1.1 ± 0.4	2	8	1.1	3.3 ± 1.9	7
	March, 2017					4	0.8	3.0 ± 2.3	5
	November, 2017	12	4.7	3.1 ± 3.3	10				
	December, 2017	14	7.8	4.6 ± 3.1	11				
	January, 2018	29	11.1	5.3 ± 7.9	39				
	February, 2018	11	4.8	4.2 ± 3.9	15				
	April, 2018	17	6.4	7.5 ± 13.8	58				
	January, 2019	2	0.2	1.5 ± 0.7	2				
	February, 2019	15	5.6	3.5 ± 2.9	10				
	March, 2019	53	20.4	3.6 ± 3.4	13				

DO _{sat}	Month	Sechura Bay				Paracas Bay			
		Frequenc y	P (%)	Duration (hr)	Max (hr)	Frequenc y	P (%)	Duration (hr)	Max (hr)
24%	January, 2012	16	1.7	1.0 ± 0.0	1	7	0.3	1.0 ± 0.0	1
	February, 2012					11	0.5	1.0 ± 0.0	1
	March, 2012	13	1.4	1.0 ± 0.0	1	1	0.1	1.0 ± NA	1
	April, 2012	3	0.2	1.0 ± 0.0	1	1	0.1	1.0 ± NA	1
	May, 2012	13	0.8	1.0 ± 0.0	1	1	0.1	1.0 ± NA	1
	June, 2012	13	5.1	1.0 ± 0.0	1				
	July, 2012	11	18.0	1.0 ± 0.0	1				
	August, 2012	10	1.6	1.0 ± 0.0	1				
	September, 2012	6	0.8	1.0 ± 0.0	1	11	1.3	2.6 ± 2.7	8
	October, 2012	4	0.5	1.0 ± 0.0	1	59	7.9	3.1 ± 2.9	13
	November, 2012	4	0.5	1.0 ± 0.0	1	100	18.6	4.2 ± 4.3	19
	December, 2012	4	0.5	1.0 ± 0.0	1	74	18.2	5.7 ± 6.8	30
	January, 2013	3	0.2	1.0 ± 0.0	1	56	20.2	8.2 ± 10.7	66
	February, 2013	6	0.6	1.0 ± 0.0	1	56	20.0	8.3 ± 8.6	39
	March, 2013	8	0.8	1.0 ± 0.0	1	59	13.5	3.6 ± 3.3	16
	April, 2013	12	0.6	1.0 ± 0.0	1	1	0.1	1.0 ± NA	1
	May, 2013	4	0.2	1.0 ± 0.0	1	3	0.2	1.0 ± 0.0	1
	June, 2013	5	2.0	1.0 ± 0.0	1	1	0.1	1.0 ± NA	1
	July, 2013	6	9.8	1.0 ± 0.0	1	1	0.1	1.0 ± NA	1
	August, 2013	2	0.3	1.0 ± 0.0	1	4	0.2	1.0 ± 0.0	1
	September, 2013	2	0.3	1.0 ± 0.0	1	1	0.0	1.0 ± NA	1
	October, 2013	4	0.5	1.0 ± 0.0	1	2	0.1	1.0 ± 0.0	1
	November, 2013	4	0.5	1.0 ± 0.0	1	5	0.2	1.0 ± 0.0	1
	December, 2013	12	1.4	1.0 ± 0.0	1	13	0.6	1.0 ± 0.0	1
	January, 2014	6	0.4	1.0 ± 0.0	1	11	0.5	1.0 ± 0.0	1
	March, 2014	5	0.5	1.0 ± 0.0	1				
	April, 2014	5	0.3	1.0 ± 0.0	1	9	0.6	1.0 ± 0.0	1
	September, 2014	4	0.5	1.0 ± 0.0	1	12	0.8	1.0 ± 0.0	1
	October, 2014	2	0.3	1.0 ± 0.0	1				
	February, 2015	8	0.8	1.0 ± 0.0	1	1	0.0	1.0 ± NA	1
	March, 2015	9	0.9	1.0 ± 0.0	1	43	19.0	6.9 ± 8.2	47
	April, 2015	2	0.1	1.0 ± 0.0	1	87	30.0	5.3 ± 5.3	22
	May, 2015	5	0.3	1.0 ± 0.0	1	112	35.2	5.0 ± 4.8	32
	June, 2015	2	0.8	1.0 ± 0.0	1	114	30.8	4.1 ± 6.5	52
	July, 2015	5	8.2	1.0 ± 0.0	1	157	47.1	4.7 ± 5.3	32
	August, 2015	1	0.2	1.0 ± NA	1	32	7.6	4.0 ± 3.6	13
	September, 2015					56	11.4	4.5 ± 4.4	19
	October, 2015					50	21.9	10.0 ± 11.2	77
	November, 2015					134	28.1	4.7 ± 6.1	35
	December, 2015					13	32.3	57.2 ± 84.0	312
	January, 2016					69	26.6	8.8 ± 14.9	80
	February, 2016	2	0.2	1.0 ± 0.0	1	67	19	6.6 ± 6.7	32
	March, 2016	6	0.6	1.0 ± 0.0	1	39	14.9	6.0 ± 5.5	22

DO _{sat}	Month	Sechura Bay				Paracas Bay			
		Frequenc y	P (%)	Duration (hr)	Max (hr)	Frequenc y	P (%)	Duration (hr)	Max (hr)
	April, 2016	8	0.4	1.0 ± 0.0	1	6	1.3	3.3 ± 2.2	7
	May, 2016	1	0.1	1.0 ± NA	1	28	5.9	3.4 ± 4.2	20
	June, 2016	1	0.4	1.0 ± NA	1	41	12.9	4.8 ± 4.6	17
	July, 2016	3	4.9	1.0 ± 0.0	1	23	5.5	3.7 ± 2.6	10
	August, 2016	5	0.8	1.0 ± 0.0	1	3	0.3	1.7 ± 1.2	3
	September, 2016	1	0.1	1.0 ± NA	1	5	0.6	2.6 ± 1.8	5
	October, 2016	6	0.8	1.0 ± 0.0	1	3	0.3	2.3 ± 2.3	5
	November, 2016	25	3.7	1.2 ± 0.6	4	8	1.6	4.5 ± 3.2	10
	December, 2016	31	4.7	1.3 ± 0.6	3	23	6.9	6.9 ± 3.7	13
	January, 2017	21	2.1	1.4 ± 0.7	3	22	5.4	5.6 ± 3.6	11
	February, 2017	27	3.6	1.3 ± 0.5	3	73	14.6	4.6 ± 5.5	35
	March, 2017	10	3.4	3.2 ± 2.7	9	5	1.0	3.2 ± 3.2	8
	April, 2017	7	2.3	6.4 ± 4.1	13	2	0.1	1.0 ± 0.0	1
	May, 2017	11	6.7	10.0 ± 9.5	33				
	August, 2017	6	3.7	3.8 ± 2.3	7				
	September, 2017	11	4.3	3.0 ± 2.2	8				
	October, 2017	30	15.3	4.1 ± 3.0	12				
	November, 2017	45	39.5	7.0 ± 7.5	32				
	December, 2017	42	67.1	13.3 ± 16.9	68				
	January, 2018	59	40.9	9.7 ± 27.6	176				
	February, 2018	17	11.0	6.1 ± 4.5	15				
	April, 2018	63	29.2	9.2 ± 20.5	159				
	May, 2018	8	1.6	3.3 ± 2.2	7	1	0.1	1.0 ± NA	1
	January, 2019	17	13.3	10.9 ± 22.6	96				
	February, 2019	103	56	5.2 ± 10.7	85				
	March, 2019	109	59.1	5.1 ± 5.1	25				
	April, 2019	6	2.2	7.3 ± 7.0	16	1	0.1	1.0 ± NA	1
	May, 2019	23	5.8	4.1 ± 5.2	25				
	June, 2019	1	1.6	4.0 ± NA	4				

2.3.3 Lethal times in *A. purpuratus*

The normoxic control treatment (without feeding) exhibited a high survival rate ($92.5 \pm 2.5\%$, 95% CI), and mortality started from the seventh day. The shortest median lethal time under severe hypoxia (1% DO_{sat}) was 40.5 hr (1.7 days) in the large-sized group, whereas it was 48 hr (2 days) in the small-sized group. Both groups exhibited different survival times (Figure 2.6a) : the Lt₅₀ was 3.9 ± 0.25 days (95% CI) for the small-sized group versus 2.7 ± 0.35 days (95% CI) in the large-sized group. Similarly, Lt₉₀ was 5.0 and 3.9 days in the small and large-sized groups, respectively. A positive and significant correlation (linear regression, $p < .0001$; $R^2 = .41$ and $.32$ in the small and large-sized groups, respectively) was found between the scallop survival time and condition index (Figure 2.6b). Although we carried out replicates at different times (e.g., 2 weeks between the first and the third replicate), there was no effect of the experiment

time (i.e., no effect of the maturation stage, gonadosomatic index and acclimation time) on survival (Wald test, $p > .05$).

2.4 Discussion

2.4.1 Environmental characterization of Sechura and Paracas Bays

Sechura and Paracas Bays exhibited similar patterns in bw temperature variations (Figure 2.2). These similarities can be explained by seasonality and the fact that both bays are under the influence of large-scale events such as ENSO. The latter was a significant factor in explaining the monthly environmental variability in both bays (Figure 2.3). This synchronicity in temporal trends extends further south within the geographic range of *A. purpuratus*: in Tongoy Bay (Chile), Ramajo et al. (2019) observed patterns in DO, pH and temperature apparently synchronous with ours during the years 2016–2017.

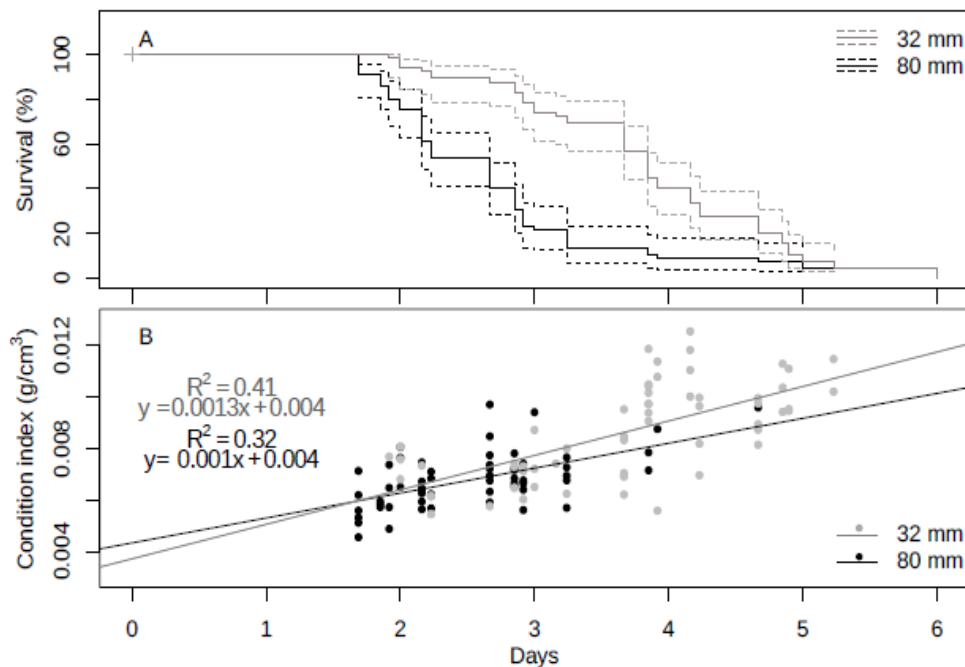


FIG. 2.6: Survival of small and large-sized groups of *Argopecten purpuratus* at 1% DO_{sat}: (a) survival curves and (b) the relationship between individual survival time and condition index. Dashed and dotted lines represent 95% confidence intervals and the median lethal time, respectively. Linear regression models represent the significant positive relationships ($p < 0.0001$) between the survival time and condition index in both size groups.

Nevertheless, Peruvian bays exhibited lower DO concentrations and pH, and fluctuations in temperature, oxygen, and pH were more abrupt than those observed in Tongoy Bay. Sechura Bay, being located in a transition zone between tropical water masses and the colder Humboldt Current, exhibited a higher variability in environmental parameters (i.e., high peaks of temperature, but low salinity due to rainfalls). Conversely, Paracas Bay, located near one of the most intense upwelling cells of Peru (Zuta Urquiza, 1972), exhibited higher DO variability and more frequent low-DO_{sat} periods. Such variability is probably due to the advection of hypoxic water masses from the upwelling system

combined to local sediment oxygen uptake (Aguirre-Velarde et al., 2019b ; Chauca-Vela, 2018 ; Ohde, 2018). Both bays exhibited long periods of occurrence of moderate hypoxia (below 24% DO_{sat}), but different relationships with other environmental variables. In Sechura, lower DO_{sat} in bw was positively correlated to lower salinity, which suggests that hypoxia in this bay is probably linked to stratification. In Paracas, low DO_{sat} was positively related to higher bw temperatures. This result seems to contradict the observations of Aguirre-Velarde et al. (2019b) for Paracas, but may be explained by the frequency in data acquisition and analysis between the two studies. The present study analyzed low-frequency data and the correlation between high temperature and low DO_{sat} can be explained by seasonality : low DO_{sat} events occurred mainly during summer when the temperature was on average higher, but also more variable (Aguirre-Velarde et al., 2019b). The analysis of high-frequency data in Aguirre-Velarde et al. (2019b) showed that hypoxia events occurred concomitantly with sudden drops in temperature in summer, suggesting the advection of upwelled cold and oxygen-depleted water. Indeed, the same trend can be observed in our high-frequency data (when available) in both bays (Figure 2.4b–e). Hypoxia thus appears as a chronic stressor in Sechura and Paracas Bays, which exhibited more than 38% of the summer time under moderate hypoxia (below 24% DO_{sat}; Table 2.2). Both bays exhibited much longer periods in hypoxia than Seatuck Cove, USA (5.3% of the time under 29.7% DO_{sat}; Gurr et al., 2018), where hypoxia has been reported as a problem for the Atlantic Bay scallop, *A. irradians*. Moderate hypoxia was also more prolonged in Sechura Bay than in Tongoy Bay (Ramajo et al., 2019) during the last 3 months of 2017.

2.4.2 Hypoxia-related mortality and size-related tolerance to hypoxia

All scallop mass mortalities in Sechura and Paracas (years 2012, 2013, 2015, 2017, and 2019) occurred during the warmer months (austral Summer and early Autumn) and were always preceded by the occurrence of severe (below 5% DO_{sat}) to moderate (below 24% DO_{sat}) hypoxia within the previous 2 weeks (Figure 2.4). Additionally, continuous moderate hypoxia was present for more than 2 days in bw before mass mortality events (Figure 2.4), which emphasizes the need of evaluating the ability of *A. purpuratus* to cope with hypoxia. Many studies stated that the adverse effects of hypoxia on aquatic organisms depend on the intensity and duration of hypoxic events (eg., Brokordt et al., 2013 ; Diaz and Rosenberg, 1995 ; Llansó, 1992). Our experimental results highlight the higher ability of *A. purpuratus* to cope with severe hypoxia compared with other pectinids : the first specimen died after 40.5 hr at continuous exposure to 1% DO_{sat} (Figure 2.6a), which is 1.4 to 3.4-fold higher than the 28, 16, and 12 hr reported in *A. irradians* (Gurr et al., 2018), *Patinopecten yessoensis* (Sakurai et al., 2000), and *Chlamys delicatula* (Mackay Shumway, 1980), respectively. This ability would be an adaptive response of the species to this recurrent selective pressure linked to the upwelling system.

Facing severe hypoxia (ca. 1% DO_{sat}), *A. purpuratus* survival was size-dependent (negative relationship ; Figure 2.6a), which agree with observations for other bivalves such as *P. yessoensis* (Lt₅₀ of 4 and 2.6 days for 8.8 and 59.4 mm size groups, respectively ; Seto et al., 2004), *Mulinia lateralis* (Lt₅₀ of 7 and 4 days for 5 and 10-mm size groups, respectively ; Shumway et al., 1983), and *Perna perna* (Lt of 19 and 12 days for 29 and 58-mm size groups,

respectively ; Hicks and McMahon, 2005). Lower tolerance (survival time) of large-sized specimens to severe hypoxia is consistent with the hypothesis that a lower surface-to-volume ratio in larger individuals confers lower ability to extract oxygen from the water (related to the surface area) in light of their oxygen requirements (at least partly related to the volume). Another non-exclusive hypothesis is that reproductive investment in larger scallops may reduce their capacity to cope with hypoxia. Indeed, Brokordt et al. (2015) observed that mature and post-spawning *A. purpuratus* are more vulnerable to hypoxia than juveniles. Such a pattern could be due to the additional energy mobilization towards spawning after maturation (Kraffe et al., 2008), which would increase oxygen demand, and/or to reduce reserves for aerobic and anaerobic metabolisms under hypoxia (Brokordt et al., 2015). The higher proportion of juveniles after mass mortalities in the years 1995 to 2014 in the natural bed of Lobos de Tierra Island (south of Sechura, Flores-Valiente et al., 2019) would agree with a higher tolerance of small-sized scallops to environmental stressors. In fine, smaller body size would be an outcome to global warming and deoxygenation of seawater (Baudron et al., 2014 ; Daufresne et al., 2009 ; Gardner et al., 2011).

In contrast, Nilsson and Östlund-Nilsson (2008) hypothesized that large-sized individuals have an advantage over the smaller ones during prolonged severe hypoxia and anoxia, when individuals rely on anaerobic ATP production for survival. The small-sized individuals would run out of energy reserves or would reach lethal levels of toxic anaerobic end-products faster than the large-sized specimens, due to their higher mass-specific metabolic rate. Indeed, the Lt_{50} of *P. perna* increased with increasing shell length (SL) under anoxia (~7 and 21 days at 20°C for 29 and 58 mm SL, respectively ; Hicks and McMahon, 2005). This feature was not so clear ; however, for *P. yessoensis* (Lt_{50} of 0.8 and 1.3 days at 15°C for 8.8 and 59 mm SL, respectively ; Seto et al., 2004). *P. perna* (Hicks and McMahon, 2005), *Mytilus edulis* (Babarro de Zwaan, 2008), and *Mytilus galloprovincialis* (de Zwaan et al., 1991) are very tolerant to anoxia. These mytilid species, frequently exposed to anoxia when their valves are hermetically closed, may have efficient anaerobic metabolism. These periods of “self-imposed” anoxia lead to hypometabolic conditions, activation of the anaerobic pathways for energy production, and an effective antioxidant system (Hermes-Lima et al., 1998) *M. edulis* and *Mytilus trossulus*, when closing their shells, maintain the pallial fluid in a hyperosmotic state for a while (Qiu et al., 2002) and may reduce sulfide diffusion. Anoxia does occur with some co-stressors, such as hydrogen sulfide and bacterial proliferation, which may reduce bivalve survival (Vaquer-Sunyer and Duarte, 2010 ; Yamada et al., 2016). The present experimental study could not be compared to the studies aforementioned since scallops were not exposed to anoxia and co-stressors were not measured in our experiments.

Interestingly, individual survival time under severe hypoxia was positively related to the condition index (CI, Figure 2.6b). Such a pattern might be explained by higher availability of energetic reserves for anaerobic metabolism in individuals presenting higher CI (Nilsson and Östlund-Nilsson, 2008). Several bivalves use the glycogen stored in all the body tissues to maintain the organism's total energy demand (Barber and Blake, 2016). Specifically, Pectinidae switch their metabolism toward anaerobic metabolism during hypoxia, forming octopine within their adductor muscle (Grieshaber et al., 1994). The

positive relationship between scallop survival and individual CI during the laboratory experiments highlights that a high CI would allow the individuals, whatever their size, to maintain their metabolism for a longer time.

2.4.3 Sustainable management of scallop aquaculture

During recent decades, the frequency, duration, and severity of hypoxic crises have increased worldwide (Diaz and Rosenberg, 2008), including Sechura and Paracas Bays. The latter has more risk of facing severe hypoxia than Sechura because milky-turquoise waters and anoxic events are frequent in summer, leading to reproduction disruptions, somatic weight losses, and increased scallop mortality Aguirre-Velarde et al. (2019b). Exposure to severe hypoxia and related environmental conditions affect scallop bioenergetics and subsequently growth and reproduction (Aguirre-Velarde et al., 2019a). Hypoxic crises mainly extend in summer and early fall, from January to April in Sechura Bay and from December to April in Paracas Bay (Table 2.3). Hypoxia during summer is associated with more stratified seawater along the Peruvian coast compared to the winter time when the water column is totally mixed and colder (Gutiérrez et al., 2011). In the last decades, scallop farming in Peru showed competitive advantages compared to Chile because of lower production costs and high availability of seeds on natural beds (Bossier, 2015 ; Kluger et al., 2019b). This may not be true anymore because environmental stressors are more frequent in Peruvian bays. The present study focused on hypoxia, but other stressors (red tides, hydrogen sulfide episodes, ocean acidification, etc.) might also affect scallop farming and lead to production losses. Unfortunately, the long-time scallop cultivation process increases the risk of mortality due to a higher probability of mismatch between environmental conditions and scallop ecophysiological requirements. The long-term cultivation of this species may also include loss of genetic diversity due to generational breeding and cause a gradual decline of natural stocks.

The failure of current scallop production strategies in Peru requires new farming modalities in order to limit summer mass mortalities in culture and economic losses for producers and the food chain industry. This is particularly true in the context of climate change and other anthropogenic pressures, which will probably lead to increased frequency of hypoxic events in the bottom water of Peruvian shallow coastal bays in the next decades. The future management plan in Sechura and Paracas Bays could include a combination of high-frequency environmental monitoring, changes in culture techniques and annual planning, ecosystem management and experimental management (with direct intervention to enhance productivity and survival rate : see Avendaño et al., 2017) : (a) Real-time and high-frequency environmental surveys would allow farmers to get an early warning on environmental changes in the culture areas. Bottom-water characteristics (temperature, DO_{sat} , salinity, hydrogen sulfide, red tides) are essential to monitor as long as bottom scallop culture remains the rule, especially in summer. Temperature records more offshore could also allow farmers to anticipate changes in the upwelling intensity and ENSO phases, in order to predict the onset of hypoxic crises. (b) The cultivation technique should be changed from bottom to suspended culture whenever possible (e.g., by using lantern nets) to avoid severe hypoxia (and likely hydrogen sulfide) peaks in bw and at the water–sediment interface. Seed collection in suspended artificial devices could be also tried out, in order to provide substrates for post-larval settlement off bottom. In addition, better growth in suspended culture (Aguirre-

Velarde et al., 2019b) would allow reducing the culture cycle duration and further decrease the scallop mortality risk. Experimental management could deal with optimal depth for seed settlement and growth, density-dependent seed survival and growth in lantern nets, and the ecological side effects of scallop production off bottom. Usually, the cost of suspended culture in lantern nets, baskets, or cages is lower than that relative to bottom culture that may require SCUBA diving (depending on depth conditions). However, one may consider the relatively small total surface area being cultivated with suspended devices (defined by the number of lantern nets) compared with bottom culture (Alcázar Mendo, 2008). Suspended culture has been well-implemented in another scallop culture area called Los Chimus Bay, with no significant mortality reports and constant production during the last years. (c) Harvesting schedules should be planned to avoid having large-sized individuals in culture during the austral summer and fall months (these months exhibit frequent severe hypoxia). The critical month of December could be the last month of harvesting, at least on the bottom. (d) In parallel with seed collection on artificial substrates, monitoring recruitment in natural beds would ensure to catch seed for the next cultivation period and likely to increase the survival rate of these juveniles when cultivated off bottom. (e) When relevant, the culture sites should be located far from freshwater inputs, especially during the rainy ENSO warm phases. Coping with climate change and in order to reduce the risk of production losses, artisanal producers should try to optimize their practices. Ecosystem management options may include habitat manipulation (e.g., by providing artificial substrates, such as Japanese devices, to replace natural ones which are located on bottoms with unsuitable environmental conditions), restocking of depleted areas (by seeding of juveniles on predator-cleaned bottom), and estimating the carrying capacity of Paracas and Sechura Bays taking into account the seasonal variability in oxic conditions. Finally and as suggested by Aguirre-Velarde et al. (2019b) ; Cisneros Burga Argüelles Torres (1996) ; Mendo et al. (2002), whether cultured scallops could be transferred in other coastal areas during hypoxic/anoxic events and co-occurring episodes of milky-turquoise waters ("aguas blancas") would also need to be evaluated in terms of technical feasibility and economic profitability, as the ultimate chance to avoid or reduce mortalities.

2.5 Conclusions

Environmental surveys conducted in this study showed that hypoxia is persistent throughout most months in Peruvian shallow coastal waters such as Sechura and Paracas Bays, with higher intensity and duration during summer and early fall. Prolonged exposure to hypoxia and other co-occurring environmental stressors may affect scallop survival. We demonstrated the importance of using the individual size in the subsequent environmental stress experiments. The present study showed that the smaller individuals (31 mm shell height) were more tolerant to severe hypoxia than the bigger ones (80 mm shell height). The positive relationship between scallop survivorship and individual condition index during the laboratory experiments highlights that a high condition index would allow the individuals to maintain their basal metabolism for a longer time. Further studies should consider survival when scallops face other single thresholds (e.g., salinity, pH, hydrogen sulfide, red tides) and most importantly the combination of multiple stressors since they may affect the tolerance and the ability of recovery of the scallops in a greater portion than single stressors.

Finally, as a consequence of global change, rising temperatures, oxygen depletion, and acidification in seawater are expected to be more frequent, so scallop managers must account for the potential risks of these environmental stressors in planning their production activities. Clearly, there is a need for site-specific management strategies in Peru due to the distinctive environmental features of the various coastal bays hosting scallop culture.

Acknowledgments

We thank the Instituto del Mar del Peru and especially the staff from the Aquatic Ecophysiology Laboratory, the Phytoplankton laboratory, and the Chemical Oceanography laboratory. We thank Lucero for their active help during the experiments. We thank to APROPRISCO for allowing us to dive in their aquaculture concession to collect the scallops used in the laboratory experiment. This work is part of a PhD thesis supported by IRD within the framework of the LMI DISCOH. Rosa Cueto-Vega is supported by a grant from the Franco-Peruvian Doctoral School in Life Sciences.

Chapter 3

Effect of individual size on oxygen regulation of *A. purpuratus* under progressive hypoxia

Frontiers (in prep.)

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Abstract

Hypoxia is a major environmental stressor in the Humboldt Current System. To investigate the respiratory response of different size-groups of the scallop *Argopecten purpuratus* increasing hypoxic conditions, oxygen uptake rate (VO_2) of individuals facing progressive decrease of oxygen saturation (from 100 to 5%) was measured in seven size batches (range between 20.5 and 77.3 mm, shell height). For the analysis purposes, batches were classified in small, medium and large size-groups (mean sizes : 25.1 ± 3.4 , 44.1 ± 4.2 and 74.0 ± 2.0 mm shell height, respectively). The response curve during progressive hypoxia was evaluated using a segmented linear regression. The break points between linear segments allowed estimating the oxygen critical points (P_{cO_2} , % O_2 saturation), while the segmented linear models were used to assess the regulatory capacity. In normoxia, average individual VO_2 were lower in the smallest than in the largest scallop batch (0.10 ± 0.007 and 2.04 ± 0.004 $mgO_2 \text{ ind}^{-1} \text{ h}^{-1}$, respectively). At 20% oxygen saturation, respiration rates were between 0.80 and 0.91-fold lower than in normoxic conditions for all the groups sizes. At 5% O_2 saturation, respiration rates were 0.51, 0.38 and 0.39-fold lower than normoxia for the small, medium and large size-group, respectively. Mean P_{cO_2} estimates were lower in smaller and medium groups (14.7 ± 1.6 and $15.5 \pm 2.1\%$, respectively) than in larger ones (20.2 ± 2.4 SD). The oxygen regulation percentage found in this study was greater than 80% within the size range studied, which corresponds to a “good regulator” species. Additionally, overshoots (abrupt increases of respiration rates) after short exposure to severe hypoxia emphasize an oxygen debt ; which was 2.2-fold greater in large scallops than in smaller ones. This study suggests that larger individuals might have a lower ability to cope with hypoxia, so might be more sensitive to this rising environmental stressor, with potential consequences for scallop aquaculture and evolutionary trends.

Keywords : Bivalves, *A. purpuratus*, respiration, severe hypoxia, tolerance thresholds.

3.1 Introduction

Respiration, which includes the transport of oxygen from the outside of the organism into the cells and the transport of carbon dioxide in the opposite direction, responds directly to metabolic needs from several processes in the organisms such as digestion/assimilation, growth, maintenance and reproduction (Kooijman, 2000). Understanding respiration under hypoxia is important to predict the effects of this stressor on life traits of marine organisms (Thomas et al., 2019). Throughout evolution, anoxic events (along with other extreme events) have been responsible for great mass extinction episodes (Jenkyns, 2010 ; Brenneke et al., 2011 ; Watson, 2016). Future scenarios for 2100 (i.e., RCP8.5) postulated that Eastern Boundary Current Systems (EBUS) would move poleward and weaken at low latitudes (Pörtner et al., 2014 ; García-Reyes et al., 2015; Bindoff et al., 2019). Reduced upwelling can induce stronger deoxygenation and low pH, which can particularly affect coastal habitats and benthic organisms in the Northern Humboldt Current System.

When facing progressive decrease of environmental oxygen saturation, aquatic organisms exhibit various abilities to regulate their respiration (Mangum Winkle, 1973 ; Taylor Brand, 1975). Organisms are able to maintain relatively constant oxygen consumption rates over a wide range of oxygen concentrations are called “oxy-regulators” ; while others whose oxygen consumption depends on the oxygen concentration in the environment are called “oxyconformers”. Exists also intermediates response to hypoxia (Mangum Winkle, 1973) thus, besides oxy-regulators behave like oxy-conformers below a critical threshold in partial pressure of oxygen (i.e. oxygen critical point P_{cO_2}). Herreid (1980) considers that “good regulators” have low P_{cO_2} values and abrupt transitions when reducing its respiration rates. “Poor regulators” are expected to have a more gradual transition from oxy-regulation to oxy-conformity. Bivalve species inhabiting periodically hypoxic habitats are generally found to be better regulators when submitted to progressive hypoxia (Griffiths and Griffiths, 1987).

The first attempts of marine organisms to cope with hypoxia are a biochemical compensation as well as stimulation of the blood circulation, increase respiratory pigments production, and increase respiration rates ; after a long exposure to hypoxia organisms reduce their energy expenditure and/or start anaerobic metabolism (Wu, 2002). The reduction of protein synthesis is a physiological compensation that allows to reduce energy expenses and oxygen consumption (i.e. metabolic depression ; Storey and Storey, 1990 ; Via et al., 1994 ; Hochachka, 1997). In the other hand, anaerobic metabolization of reserves (e.g. carbohydrates) to fuel all or a part of the organism’s total energy demand is less efficient and may have deleterious consequences due to the accumulation of toxic anaerobic metabolites (Herreid, 1980). In bivalves, short time exposure to anoxia followed by a re-oxygenation frequently results in an overshoot of oxygen consumption highlighting an apparent oxygen debt (Herreid, 1980). This oxygen debt may be related to the restoration of aerobic metabolism which means the restoration of high-energy phosphate and the synthesis of glycogen from accumulated succinate during the early period of recovery from anoxia (Shick et al., 1988).

The Peruvian scallop, *Argopecten purpuratus*, is a hermaphrodite pectinid bivalve, found along the Pacific coast of Peru and northern Chile (Wolff and Mendo, 2000) in semi-protected coastal and island bays with soft sediment

(Brand, 1991). The Peruvian coast belongs to one of the most productive Eastern Boundary Upwelling Systems of the world, the Humboldt Current System, it is characterized by degradative processes of settling organic matter that consume oxygen in deep waters and on the bottom (Gewin, 2010 ; Bertrand et al., 2011). Past environmental monitoring has revealed that coastal bays, where Peruvian scallop aquaculture takes place, are facing chronic hypoxic conditions (Aguirre-Velarde et al., 2019b). For instance, an important variability of oxygen saturation has been shown in Paracas Bay, and oxygen saturation can be, for long periods, below the known oxygen uptake regulation ability of *A. purpuratus* (Aguirre-Velarde et al., 2016). The respiration of *A. purpuratus* under hypoxia has been documented in several studies ; in particular, Aguirre-Velarde et al. (2016) found a critical point of 24.4%, independent of temperature. The effects of environmental hypoxic events on scallop populations depend on the intensity and duration of these events (Llansó, 1992). Hypoxia exposure also affects the escape capacity of *A. purpuratus*, thus increasing vulnerability to predation and decreasing physiological capabilities to support other stress factors (Brokordt et al., 2013).

Chronic exposure to hypoxic conditions may have negative consequences on Peruvian scallop cultures by changing growth, reproduction and survival responses (Aguirre-Velarde et al., 2019b). It has recently been shown that survival of *A. purpuratus* is size-dependent with small individuals showing a longer survival time under extreme hypoxia (ca. 1% O₂ saturation) than the biggest ones (Cueto-Vega et al., 2021, Chapter 2). Thus, individual size may play a role in the ability to regulate oxygen consumption and to detoxify metabolites after reoxygenation of the seawater. Because body size affects energetics, increasing body size has implications for resilience in the face of climate change (Gardner et al., 2011). Indeed, increasing body size results in a reduction of the surface-to-volume ratio, which would consequently lead the large organisms to become more oxygen limited (Wang and Widdows, 1991 ; Robb and Abrahams, 2003). Besides, a lower surface-to-volume ratio in larger individuals signifies higher body weight and thus higher net energetic demand. In other bivalve species, the respiratory response to progressive hypoxia and its recovery were found to be size-dependent : studies in *Crassostrea gigas* larvae (Widdows, 1989), *Mytilus edulis* larvae (Wang and Widdows, 1991) and *Perna perna* (adults and juveniles, Hicks and McMahon, 2002) showed that the ability to regulate oxygen consumption increases with decreasing individual size.

Because they are easier to manipulate in the laboratory, most previous studies have been performed on small individuals. As a consequence, size remains an underestimated variable when considering exposure to hypoxia. Despite its importance for ecology and sustainable aquaculture management, there are few studies dealing with the effect of individual size on the respiratory response and recovery of individuals facing hypoxia. This study aims at better understanding of respiratory rates at decreasing oxygen levels of different sizes of *A. purpuratus* processes of recovery after 12 hours of exposure to severe hypoxia (5% oxygen saturation). The present work also allows to better define the variability of hypoxia-tolerance thresholds with size. In the coming years, hypoxic crises will become more frequent and intense (Diaz and Rosenberg, 2008 ; Stramma et al., 2010), so the degree of adaptation of marine organisms to such events may be critical for sustainability for species from coastal waters, especially those from the intertropical EBUS.

3.2 Materials and Methods

3.2.1 Sampling and acclimation procedure

In total, seven different individual batches with various sizes (ie. shell height) were used in this study ; all scallop specimens were collected by scuba diving at different sampling times. In November 2017, large- (L1: 75 ± 2 mm SD, shell height) and medium-sized *A. purpuratus* (M : 38.7 ± 2 mm SD, shell height) were collected in Paracas Bay (Atenas beach, Peru; $13^{\circ}50'35.8''\text{S}$, $76^{\circ}15'59.5''\text{W}$). In February 2018, samples of small scallops (S : 20 ± 2 mm SD, shell height) were collected from Casma (Los Chimos beach ; $9^{\circ}19'42.5''\text{S}$, $78^{\circ}28'15.4''\text{W}$) and large scallops (L2: 77 ± 2 mm SD, shell height) were sampled from Callao (San Lorenzo Island coas ; $12^{\circ}01'59.1''\text{S}$, $77^{\circ}12'19.5''\text{W}$). Finally, 200 large (L3: 69 ± 2 mm SD, shell height), medium (M2 : 48 ± 2 mm SD, shell height) and small (S2: 28 ± 2 mm SD, shell height) individuals were collected from Callao in March 2019 (see location here above). Once collected, all scallops were quickly transported in a cool box to the Aquatic Ecophysiology Laboratory at the IMARPE (Callao, Peru) where they were placed into a 400-L tank for acclimation, with a flow-rate of 8 L h of 1- μm filtered seawater. Tank seawater was thermoregulated ($16.1^{\circ}\text{C} \pm 0.5$ SD) aerated (oxygen saturation above. 90%) and salinity was constant 34.9 (± 0.2 SD). During acclimation, specimens were fed a mix of cultured microalgae (*Isochysis galbana* : *Chaetoceros calcitrans* 1 :1) three times a day to maintain an average concentration of 9200 cells mL in the tank. Scallops were acclimatized for two or three weeks, except the first batch from Paracas, which was maintained in the laboratory for four months due to technical issues before starting experimentation.

3.2.2 Experimental system

We used an automated system in order to measure continuously oxygen consumption of scallops placed under progressive hypoxia. This system has been fully described in Artigaud et al. (2014) and used in Aguirre et al. (2016). Briefly, the system consisted of six acrylic chambers (0.59 L) placed in a 400-L tank (Fig. 3.1). Each chamber was equipped with two pumps and an oxygen probe (FDO 925-3 dissolved oxygen probe, WTW, USA) connected to an oximeter (Multi 3430, WTW, USA) that automatically recorded oxygen concentration within the chamber every 30 seconds. One pump allowed a continuous homogenization of the water within the chamber. A second pump allowed renewal of water within the chamber ; it was automatically activated for 5 min every 15 min, thus allowing cycles of a 15-min incubation followed by a 5-min water renewal during all the experiments. For all experiments, five out of the six chambers contained live scallops ; the sixth chamber was used as a control and only contained empty valves to account for the microbial respiration. Oxygen concentration within the experimental tank was controlled by nitrogen injection in a gas exchanger fitted in the experimental tank. An oxygen probe (FDO 925-3 dissolved oxygen probe, WTW, USA) interfaced with a computer allowed to set up the oxygen concentration within the tank at any desired value.

3.2.3 Respiration measurement procedure under progressive hypoxia

Individuals were classified into three size groups (small, lower than 30 mm ; medium, 35-50 mm ; and large, higher than 60 mm ; details in Table 3.1). The number of individuals in each of the five chambers were four, two and one for small, medium and large size groups respectively. The sixth chamber was used as a control, with the same number and size of empty valves as those of the live individuals in the five other chambers. Before each experiment, scallops were gently cleaned to remove fouling and were transferred to the chambers. All experiments were performed at 16°C. Individuals were starved one day before the beginning of the experiment. Before each experiment, chambers and oxygen probes were washed with a diluted hydrogen peroxide solution to limit bacterial development within the chambers. Once individuals were installed in the chambers, respirometric measurements started. O₂ saturation within the experimental tank was maintained close to 100% for 4 h, then the system was programmed to progressively decrease the oxygen saturation. This decrease was performed in two-hour steps at 80, 70, 60, 50, 40, 30, 20, 15, 10 and 5% O₂ saturation. Gas exchanges with the atmosphere were limited by placing a polystyrene sheet floating on the water surface. Measurements under progressive hypoxia were replicated at least three times for each scallops batch. The time lapse between replicates of each batch was about 2-4 days. At the end of each experimentation, shell height (in mm), weight, and volume of each individual were measured. Thereafter, the flesh of each specimen was excised and dried to constant weight at 65°C. In total, we obtained 87 measurements of oxygen consumption rate (under progressive hypoxia) on a total of 175 specimens (according to the number of individuals per chamber mentioned before) ; the replicates by batch are detailed in Table 2.1.

3.2.4 Recovery after severe hypoxia

Respiratory activity after short exposition to severe hypoxia, was explored on a subsample of two replicates from two batches (from S2 and L3, see Table 3.1). The experiment consisted in a prolongation of the experiment keeping the individuals 12 hours under 5% O₂ saturation, followed by a re-oxygenation to 100% oxygen saturation for 10 hours.

3.2.5 Respiration rates estimation

For each incubation (the period between two water renewals), the individual respiration rate (VO₂, mgO₂ h⁻¹ ind⁻¹) was calculated for each chamber by subtracting the oxygen consumption rate in chambers with the control, as:

$$VO_2 = \frac{Slope_{cham} \times Vol_{cham} - Slope_{cont} \times Vol_{cont}}{n_{ind}}$$

Where the Slope_{cham} and Slope_{cont} (mg O₂ h⁻¹ ind⁻¹) are slope values of the linear regression for oxygen concentration values over the time of each incubation in the chambers with scallops and in the chamber with empty valves, respectively. Vol_{cham} and Vol_{cont} are the available volume (L) of seawater in the chamber and the control, respectively, each estimated from the total volume of the chamber less the volume of live scallops or empty valves. While, n_{ind} is the

number of specimens in the chamber (4, 2 or 1 for small, medium and large group-size, respectively). Only incubations with negative slope and R^2 higher than 0.9 were retained for further analysis.

Because oxygen saturation within the experimental tank at the beginning of the experiment was generally close to 90%, respiration rate at 100% oxygen saturation (VO_2^{100}) was estimated using a linear relationship between O_2 saturation and VO_2 in the first 6 hours (oxygen saturation between 80 and 100%). The parameters of the allometric function between VO_2^{100} and dry weight (DW) were estimated using the whole dataset. To allow comparison between different batches, the respiratory response of each batch was scaled as a percentage of the batch's VO_2^{100} (VO_2^{st100}). The fold-change between VO_2 (at 5 and 20% oxygen saturation) and VO_2^{st100} was used to examine the reduction in respiration rates between the size batches after progressive hypoxia. The same standardization has been applied of respiration rate measured during recovery from severe hypoxia.

3.2.6 P_cO_2 and regulation percentage

All data treatments and subsequent statistical analyses were performed using the R software (version 3.4.4 ; R Core Team, 2021). P_cO_2 and oxygen regulation percentage was estimated using the data from the progressive hypoxia experiment. The breakpoints, considered as an estimate of the P_cO_2 (Artigaud et al., 2014), were estimated for each scallop batch using a segmented linear model through the “Segmented” R package (Muggeo, 2008). The oxygen regulation percentage, as defined by Hicks and McMahon (2002), was calculated as the ratio between the area under the O_2 saturation (%) vs standardized oxygen consumption rate (VO_2^{st100} , %) curve and the value that would be obtained for a perfect regulator (displaying a respiration rate of 100% whatever the level of oxygen saturation). The percentage estimates were performed for the whole response (as indicated before) but also for the two parts of the segmented model: part 1 (% O_2 saturation - break point) and part 2 (break point - 100% O_2 saturation). For this purpose the percentage regulation of each part was computed as the ratio between the area below the considered segment (either first or second) and the area that would realize a perfect regulator within this part (which corresponds to 100 P_cO_2 for part 1 and 100 (100 - P_cO_2) for part 2).

P_cO_2 which were compared using the 95% confidence level given by the segmented model of each size batch. To improve comparison between batches, the segmented model was used to estimate at some possible oxygen saturation thresholds : P_cO_2 (VO_2^{Pc} or respiration under moderate hypoxia), 20 (VO_2^{20}), and 5% (VO_2^5 or respiration under severe hypoxia). Then, a principal component analysis (PCA) was used to explore the relationship between potential descriptors of O_2 regulation (site, log of dry weight, break point, model's slopes, oxygen percentage regulation, VO_2^{100} and MR^{100}) and scallop size groups. All variables were centered and scaled and the PCA performed using the “factoextra” R package (Kassambara and Mundt, 2020).

3.2.7 Oxygen debt estimation

Additionally, to quantify a possible oxygen debt after 12 hours of exposure to severe hypoxia, the evolution in time of standardized oxygen consumption (VO_2^{st100}) of each tested batch were smoothed using a local regression (loess R function with a span of 0.15). Then, the oxygen debt was estimated as the

overshoot area above standardized respiration rate in normoxia (100%). These areas are shown in Fig. 3.3.

3.3 Results

3.3.1 Variability of respiration rates with size in normoxia

The estimated allometric function linking at 100% DO saturation (normoxia) and flesh dry weight was $VO_2^{100} = aDw^{0.768}$, with $R = 0.94$ ($p < 0.05$). The mean respiration rates under normoxia in the lowest size (S1) was $0.1 \text{ mgO}_2 \text{ ind}^{-1} \text{ h}^{-1}$ (metabolic rate : $0.996 \text{ mgO}_2 \text{ ind}^{-1} \text{ h}^{-1} \text{ g}^{-1} \text{ DW}$), while in the largest size (L2) it was $2.04 \text{ mgO}_2 \text{ ind}^{-1} \text{ h}^{-1}$ (metabolic rate: $0.389 \text{ mgO}_2 \text{ ind}^{-1} \text{ h}^{-1} \text{ g}^{-1} \text{ DW}$; more details in Table 3.1).

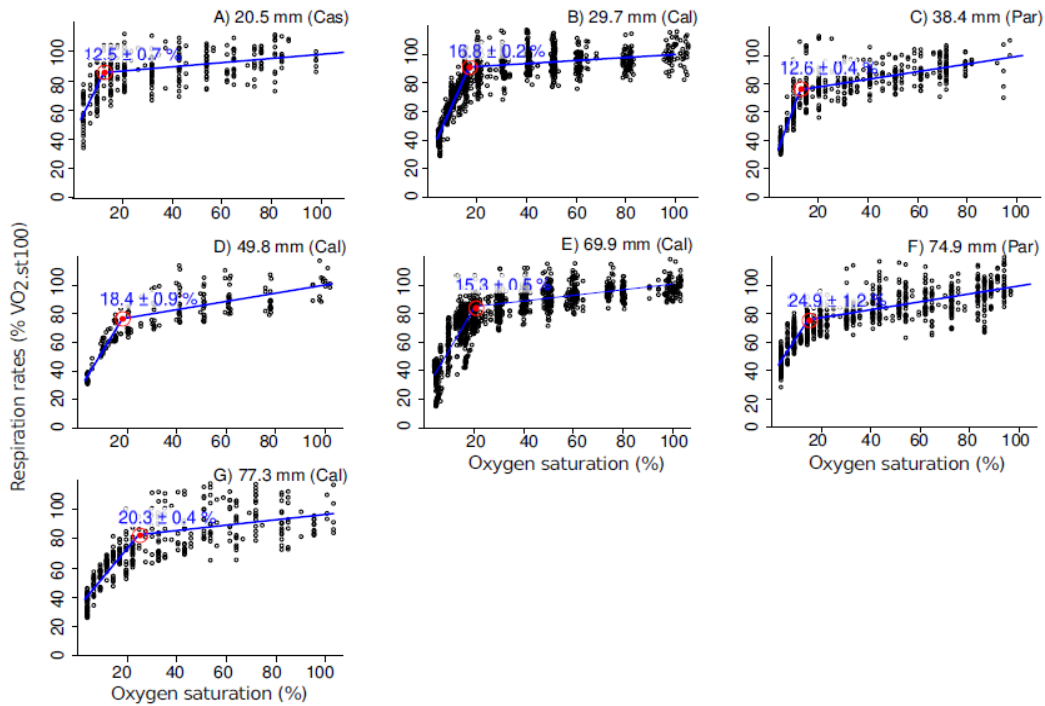


FIG. 3.1: Respiration rates (expressed as percentages of in normoxia) at 16°C of seven size batchess (20.5, 29.7, 38.4, 49.8, 69.9, 74.9 and 77.3 mm mean shell height) of *Argopecten purpuratus* as a function of oxygen saturation. Blue lines represent linear segmented models fitted to the values (see text for details). Red circles indicate the estimated P_{CO_2} . Collection sites are indicated in parenthesis as Cal, Par and Cas ; for Callao, Paracas and Casma, respectively.

TAB. 3.1: Summary of biometry and respiratory descriptors of *Argopecten purpuratus* along size batches.

Size group	Batch	Origin	SH (mm)	DW (g)	N	VO ₂ ¹⁰ ₀	VO ₂ ^P _c	VO ₂ ² ₀	VO ₂ ⁵	MR ¹⁰⁰	MR ^{Pc}	MR ²⁰	MR ⁵
Small	S1	Casma	20.5 ± 0.1	0.10 ± 0.01	11	0.10	0.08	0.09	0.05	1.00	0.80	0.85	0.55
	S2	Callao	29.7 ± 0.1	0.35 ± 0.02	15	0.29	0.26	0.28	0.14	0.83	0.74	0.80	0.40
Medium	M1	Paracas	38.4 ± 0.1	0.42 ± 0.01	11	0.31	0.26	0.26	0.13	0.75	0.62	0.63	0.30
	M2	Callao	49.8 ± 0.2	1.25 ± 0.01	3	1.02	0.86	0.78	0.37	0.81	0.69	0.63	0.30
Large	L1	Paracas	74.9 ± 0.2	2.34 ± 0.01	12	1.44	1.04	1.24	0.60	0.61	0.44	0.53	0.26
	L2	Callao	77.3 ± 0.2	5.25 ± 0.04	14	2.04	1.52	1.44	0.74	0.39	0.29	0.27	0.14
	L3	Callao	69.9 ± 0.2	3.60 ± 0.00	21	1.70	1.48	1.46	0.65	0.47	0.41	0.41	0.18

SH: shell height; DW: Flesh dry weight; N: number of measurements under progressive hypoxia.

All the VO₂ (oxygen consumption rates, mgO₂ ind⁻¹ h⁻¹) and metabolic rates (MR, mgO₂ ind⁻¹ h⁻¹ g⁻¹) were estimated at 100%, P_cO₂ (estimated in this study, see details in Table 3.2), 20% and 5% oxygen saturation using the segmented linear model of each batch.

TAB. 3.2: Estimation of the parameters of segmented linear regressions calculated for respiration rate as a function of oxygen saturation according to scallop size classes.

Size group	Batch	Size (mm)	Break point (%)	Part 1		Part 2		Total Reg. (%)
				Slope	Reg. (%)	Slope	Reg. (%)	
Small	S1	20.5 ± 0.1	12.5 ± 0.7	3.81 ± 0.42	63.1	0.15 ± 0.03	93	88.7
	S2	29.7 ± 0.1	16.8 ± 0.2	3.91 ± 0.09	58.6	0.11 ± 0.01	95.6	89.3
Medium	M1	38.4 ± 0.1	12.6 ± 0.4	4.93 ± 0.31	45.5	0.28 ± 0.02	87.9	82.3
	M2	49.8 ± 0.2	18.4 ± 0.9	2.97 ± 0.21	47.8	0.30 ± 0.03	87.8	80.6
Large	L1	74.9 ± 0.2	15.3 ± 0.5	2.81 ± 0.13	53.1	0.30 ± 0.02	87.4	81.6
	L2	77.3 ± 0.2	24.9 ± 1.2	2.11 ± 0.13	58.2	0.19 ± 0.03	92.6	83.6
	L3	69.9 ± 0.2	20.3 ± 0.4	2.94 ± 0.07	54.5	0.19 ± 0.02	92.2	84.6

Notes: The table indicates the slopes and confidence intervals of the linear segments below (Part 1) and above the P_cO₂ (Part 2), and the oxygen regulation rates (Reg. %). Standard errors are given except for the breakpoints (95% confidence intervals).

3.3.2 Variability of respiration rates with size under hypoxia

The respiratory response of each scallop batch facing progressive hypoxia is shown in Table 3.1. Mean respiration rates under oxygen saturation close to

100% were $0.2 \text{ mgO}_2 \text{ ind}^{-1} \text{ h}^{-1}$ ($\pm 0.1 \text{ SD}$; metabolic rate : $0.9 \text{ mgO}_2 \text{ ind}^{-1} \text{ h}^{-1} \text{ g}^{-1}$), $0.7 \text{ mgO}_2 \text{ ind}^{-1} \text{ h}^{-1}$ ($\pm 0.3 \text{ SD}$; metabolic rate : $0.8 \text{ mgO}_2 \text{ ind}^{-1} \text{ h}^{-1} \text{ g}^{-1}$) and $1.7 \text{ mgO}_2 \text{ ind}^{-1} \text{ h}^{-1}$ ($\pm 0.2 \text{ SD}$; metabolic rate : $0.5 \text{ mgO}_2 \text{ ind}^{-1} \text{ h}^{-1} \text{ g}^{-1}$) for the small ($25.1 \pm 3.4 \text{ mm}$), medium ($44.1 \pm 4.2 \text{ mm}$) and large ($74.0 \pm 2.0 \text{ mm}$) size-group, respectively. As described above, there was high variability under normoxia in the medium and large group size, this is best presented graphically across oxygen saturations greater than 30% (see Fig. 3.1). At 20% DO saturation, respiration rates were $0.2 \text{ mgO}_2 \text{ ind}^{-1} \text{ h}^{-1}$ ($\pm 0.1 \text{ SD}$), $0.5 \text{ mgO}_2 \text{ ind}^{-1} \text{ h}^{-1}$ ($\pm 0.2 \text{ SD}$) ; and $1.4 \text{ mgO}_2 \text{ ind}^{-1} \text{ h}^{-1}$ ($\pm 0.1 \text{ SD}$) for the small, medium and large size-group, respectively ; within 0.80 and 0.91-fold lower than in normoxic conditions. At 5% DO saturation, respiration rates were $0.1 \text{ mgO}_2 \text{ ind}^{-1} \text{ h}^{-1}$ ($\pm 0.0 \text{ SD}$; 0.51-fold lower than normoxia), $0.2 \text{ mgO}_2 \text{ ind}^{-1} \text{ h}^{-1}$ ($\pm 0.1 \text{ SD}$; 0.38-fold lower than normoxia) ; and $0.7 \text{ mgO}_2 \text{ ind}^{-1} \text{ h}^{-1}$ ($\pm 0.1 \text{ SD}$; 0.39-fold lower than normoxia) for the small, medium and large size-group, respectively.

The segmented linear regressions indicate that all scallop batches had break points (ie. P_{cO_2}) ranging between 12.5% and 26.1 % depending on size. P_{cO_2} mean estimates suggest differences between large ($20.2 \pm 2.4 \text{ SD}$) and the other size-groups (14.7 ± 1.6 and 15.5 ± 2.1 for the small and medium group, respectively). The two lowest mean P_{cO_2} were measured for scallops batches of 20.5 mm and 38.4 mm shell height (S1 : $12.5\% \pm 0.7 \text{ SE}$ and M1 : $12.6\% \pm 0.4 \text{ SE}$, respectively) corresponding to small and medium size-groups, respectively. By contrast, the two highest mean P_{cO_2} were recorded for large-sized batches (L3 : $20.3\% \pm 0.4 \text{ SE}$ for scallops of 69.9 mm shell height and L2 : $24.9\% \pm 1.2 \text{ SE}$ for scallops of 77.3 mm).

In part 1 of segmented linear regressions (DO saturation values below the P_{cO_2}), O_2 regulation means in small group was $60.9\% (\pm 1.7 \text{ SE})$, which is higher than $46.7\% (\pm 0.8 \text{ SE})$ and $55.3\% (\pm 1.4 \text{ SE})$ for the medium and large groups, respectively. O_2 regulation capacity in part 2 of the regressions were quite similar at smaller, medium and large groups ($94.3\% \pm 1.0 \text{ SE}$; $87.9\% \pm 0.1 \text{ SE}$ and $90.7\% \pm 1.6 \text{ SE}$, respectively). Total O_2 regulation capacity for smaller, medium and large groups ranged within 80.6-89.3% (details Table 3.2).

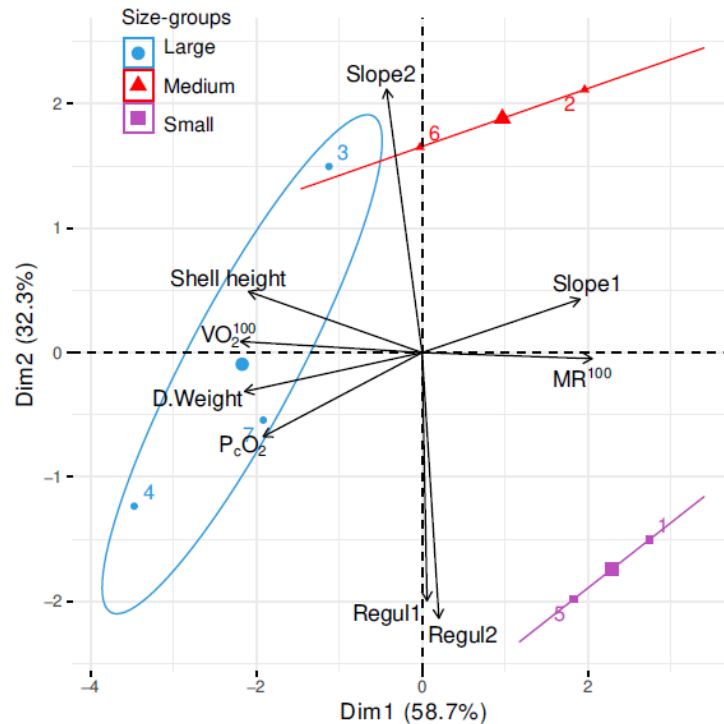


FIG. 3.2: Principal component analysis (PCA) highlighting the relationships between respirometry descriptors following progressive hypoxia. flesh-dry weight (D.Weight), and MR are recorded in Table 3.1. Breakpoints, O₂ regulation (Regul) and slopes from Part 1 and 2 are recorded in Table 3.2. The first and second principal components of the PCA contributed 55.7 and 31.2% to the total variability, respectively. Ellipses indicate 95% confidence limits of the size groupings (large, medium and small).

3.3.3 Potential descriptors explaining oxy-regulation

PCA analysis between scallop size classes after 24 hours of progressive hypoxia showed that the first and second principal components contributed 55.7 and 31.2% to the total variance, respectively (Fig. 3.2). The relationships between potential descriptors explaining oxy-regulation (site, size, Ln of dry weight, breakpoint, model slope, oxygen percentage regulation, VO_2^{100} and MR^{100}) differed according to scallop size ; size-related groupings (large, medium and small) are shown in Fig. 3.2 ; the response of the small ones is the opposite of that of the medium and large-size groups. The first component highlighted larger specimens being correlated with higher weights, higher VO_2^{100} and higher breakpoints, while smaller sizes were related with higher metabolic rates and higher regression slope (part 1) under progressive hypoxia. The second component highlighted an inverse relationship between O₂ regulation and slope 2 of the segmented regression. This component also evidenced that smaller scallops were correlated with higher O₂ regulation.

3.3.4 Respiration rate during recovery from hypoxia

After 12 h under severe hypoxia at 5% O₂ saturation, an abrupt increase in respiration rate was observed during the re-oxygenation phase. On average, standardized respiration rates reached levels higher than those found in

normoxia (Fig. 3.3). This recovery trend was observed in the two size batches (S2 and L3), but its intensity and duration depended on size. Smaller individuals exhibited an oxygen consumption rate 1.3-fold (considering loess smoothing) higher than respiration in normoxia. The increase in respiration rate for the larger size was quite similar (1.4-fold increase ; Fig. 3.3). Time to recover, i.e. to go back to VO_2^{100} , was longer for the large size batch (7 h) than for the small one (4 h). The O_2 debt by time (VO_2^{st100} by hour) was 71.8 (%. h) and 157.6 (%. h), in the small and large batches, respectively. Thus the former had a 0.45-fold lower oxygen debt than the large size-group.

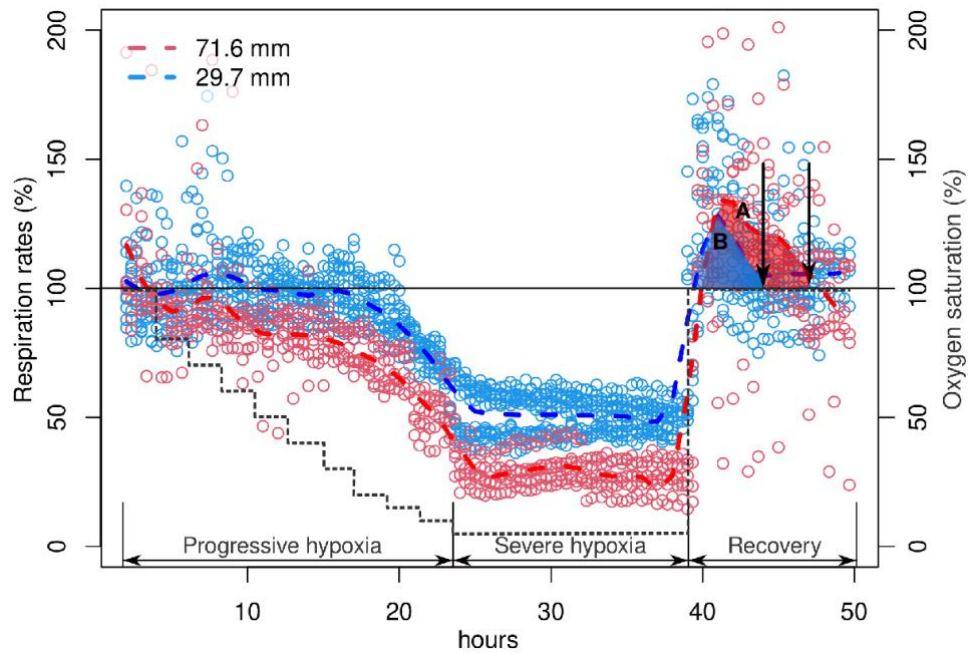


FIG. 3.3: Standardized respiratory rates (%) before and after reoxygenation for large-sized (red points) and small-sized (blue points) batches at 16°C. Blue and red lines were smoothed by using local regressions (loess R function with a span of 0.15). Vertical arrows indicate recovery at normal values of VO_2 . Red (A) and blue (B) areas are the oxygen debt estimates during recovery for the large and small size, respectively. The gray line indicates the oxygen saturation conditions throughout the experiment.

3.4 Discussion

This study evidenced that smaller scallops were better oxy-regulator than the larger ones, which is consistent with the observation from Hicks and McMahon (2002), who also found a higher oxy-regulation ability under progressive hypoxia in smaller specimens of *P. perna*. Similarly, some studies on oyster larvae (*Crassostrea gigas*, Widdows, 1989) and mussel larvae (*Mytilus edulis*, Wang and Widdows, 1991) showed that the capacity to regulate oxygen consumption would increase with decreasing individual size. The latter results are also consistent with a recent study showing that smaller individuals of *A. purpuratus* (31 mm shell height) could survive longer under extreme hypoxia (1% DO saturation) than the larger ones (80 mm ; Cueto-Vega et al., 2021). A greater ability to regulate O consumption in small-sized *A. purpuratus* to bigger ones may be explained by their relatively higher surface-to-volume ratio, i.e. a larger surface for gas exchange, and lower total oxygen requirements (related to their lower individual volume or weight). Observations in the field relating the individual size

of scallops to its ability to cope with hypoxia conditions are scarce ; nevertheless, Aguirre-Velarde et al. (2019b) reported that 50% of bottom-cultured scallops died after chronic and severe hypoxia in Paracas Bay, showing slight size-related mortality (higher survival of scallops in the size range 40-50 mm compared to 60-90 mm). This pattern is also consistent with a field study in the Chilean coast in spring 2019, characterized by intense cooling and deoxygenation events, where the adults of the species showed lower physiological rates (growth and gross calcification rates) than juveniles (Ramajo et al., 2022).

To maintain respiration rate when oxygen availability decreases, bivalves can respond by increasing water pumping/ventilation, the rate of blood perfusion and cardiac activities (Herreid, 1980 ; Grieshaber et al., 1994 ; Tran et al., 2000). This set of responses can be considered as an “oxy regulation effort”, when respiration rates are independent of water oxygen levels, the P_{cO_2} represents the hypoxic limit of this regulatory ability (Grieshaber et al., 1988). Herreid (1980) considered that “good regulators” have low P_{cO_2} values, abrupt transitions and steep conformity slopes. Meanwhile, “poor regulators” are expected to have a more gradual transition from regulation to conformity. By their part, Hicks and McMahon (2002) classified that species within 80–89% O_2 regulation are good regulators. In our study, the slopes of the segmented regression and the O regulation percentages (Fig. 3.1 ; Table 3.2) suggest that *A. purpuratus* good oxy-regulator whatever the individual size. Our P_{cO_2} values ($P_{cO_2} 16.8 \pm 0.2\%$; 16°C ; $29.7 \pm 0.1\text{mm}$) were lower than those estimated from *A. purpuratus* ($P_{cO_2} 24.4 \pm 1.9\%$; 16°C , size 24 to 32mm ; Aguirre-Velarde et al., 2016) and *Pecten maximus* a ($P_{cO_2} 23.8 \pm 1.8\%$; 18°C , size 32.5 mm ; Artigaud et al., 2014) of similar sizes. While our O regulation estimates (89.3 %) were greater than those from *A. purpuratus* (74% ; Aguirre-Velarde et al., 2016) and *P. maximus* at (84.9 \pm 6.1%, estimated only under oxy-regulation ; Artigaud et al., 2014). The good ability to regulate its oxygen consumption involves a rather large temperature range (16°C and 25°C ; Aguirre-Velarde et al., 2016). Bivalve species inhabiting periodically hypoxic habitats are generally found to be better regulators than species living in generally normoxic conditions, when facing progressive hypoxia (Griffiths and Griffiths, 1987). This rule applies to *A. purpuratus* Peruvian coastal bays, the former being frequently exposed to chronic and severe hypoxic events, especially in Summer (Aguirre-Velarde et al., 2019b ; Cueto-Vega et al., 2021).

Compared with other bivalves reviewed in Hicks and McMahon (2002), *A. purpuratus* presents a higher O_2 regulation ability, but this may not necessarily be an advantage in terms of survival. Indeed, bivalves such as *P. perna*, *Mytilus edulis*, *Arctica islandica* and *Macoma balthica* (who are poor or none regulators under progressive hypoxia ; Hicks and McMahon, 2002, and references herein) can survive longer (lethal time greater than 12 days) under extreme hypoxia (<2% oxygen saturation; Dries and Theede, 1974 ; Hicks and McMahon, 2005 ; Babarro and de Zwaan, 2008) than *A. purpuratus* (lethal time of 2.5-3.9 days at 1% DO saturation; Cueto-Vega et al., 2021). Such tolerance to prolonged anoxia has been associated with a hypometabolic condition, activation of anaerobic pathways for energy production, and an efficient antioxidant system (Hochachka and Somero, 2002). In this study, scallop inability to maintain the oxygen consumption rate below the P_{cO_2} (Table 3.2, part 1 of the model) suggests the initiation of energetic anaerobic pathways (Grieshaber et al., 1988 ; Pörtner and Grieshaber, 1993). While many bivalves are adapted to survive periodic hypoxia (Le Moullac et al., 2007), less efficient energy production under anaerobic

conditions can affect their energetics. Furthermore, the accumulation of end products, metabolized when normoxic conditions resume, can generate an oxygen debt (Herreid, 1980).

Metabolic rate, the rate at which organisms take up, transform, and expend energy and materials, is the most fundamental biological rate. Metabolic rate varies with body size and temperature by setting resource uptake rates and resource allocation from the environment for survival, growth, and reproduction (Brown et al., 2004). In this study and as for VO_2 , metabolic rates above P_{cO_2} remained close to its values under normoxia (Table 3.1), while they were ca. 80% lower than the latter at 20% DO saturation. Metabolic rate decreases as the individuals grow (Norin and Gamperl, 2018). Therefore, metabolic rate (which depends on weight) is not a good standardization tool unless measurements are made on a short term (less than few days) to avoid any growth effect. Standardization would require using a standard size, weight or the VO_2^{100} (i.e. respiration under normoxic conditions) as references. Moreover, the exponent of the relationship between the weight and respiration rate was between values expected (ca. 0.75) for ectotherms (Norin and Gamperl, 2018). By comparison, VO_2 values in normoxia in small-sized scallops from Callao and Paracas in this study are consistent with values measured by Aguirre-Velarde et al. (2016) for juvenile individuals from Paracas. Reduced P_{cO_2} in scallops from Paracas (Fig. 3.2) maybe due to a longer laboratory acclimation phase (Table 3.1), when comparing respiration rates of large *A. purpuratus* from Paracas (VO_2^{100} : 1.4 mgO₂ ind⁻¹ h⁻¹; L1) and Callao (VO_2^{100} : 1.7 mgO₂ ind⁻¹ h⁻¹; L3). If VO_2^{100} variability in large scallops is compared, the effect of acclimatization time could be greater than the effect of origin, this may be because the unrestricted transport of wild scallop seeds between Peruvian bays (Mendo et al., 2008) has produced a high local genetic variability but not a significant population structuring at the local level along the Peruvian coast (Marín et al., 2013). Another clue that may explain the high variability in VO_2 in larger Peruvian scallops is that the reproductive investment may reduce the individual response-to-stress capacity. Mature and post-spawning scallops would be more vulnerable to environmental stressors such as hypoxia than immature individuals (Brokordt et al., 2015; Salgado-García et al., 2020).

The oxygen critical point is the point below which an oxy-regulator becomes an oxy-conformer (Grieshaber et al., 1988); from a metabolic perspective, this threshold reflects a metabolic change from aerobic to anaerobic pathways to produce energy (Pörtner and Grieshaber, 1993). Depression of metabolic rate and switching to anaerobic metabolism are the two main ways allowing intertidal invertebrates to survive during anoxic conditions (Sobral and Widdows, 1997; Greenway and Storey, 2000; David et al., 2005). Decreasing metabolism is the first line of defense in highly tolerant species : metabolism can be decreased to less than 10% of basal metabolic rates (Greenway and Storey, 2000 ; Ortmann and Grieshaber, 2003 ; Larade and Storey, 2007). As observed in our study (Fig. 3.2), respiration rate decreased abruptly at 5% below the P_{cO_2} . Such drops in metabolic rate may reduce the amount of energy (ATP) needed to maintain normal functioning. Once metabolism has decreased, anaerobic metabolic pathways may be utilized to produce energy when there is not enough oxygen to maintain glycolysis (Grieshaber et al., 1994). During anaerobic metabolism, glucose is converted to pyruvate and then it can combine with an amino acid to produce fermentation end products such as octopine, analopine, strombine, or

D-lactate (Hochachka and Somero, 2002). In mollusks under hypoxia, octopine dehydrogenase (ODH) is present and more accumulated than the lactate dehydrogenase (LDH ; Grieshaber et al., 1994). This suggests that *A. purpuratus* uses both metabolic depression and anaerobic metabolism below the P_{cO_2} .

Higher respiration rates after 12 hours of severe hypoxia suggest the compensation of an oxygen debt during the posthypoxic period. This debt was evidenced by an elevated oxygen consumption (overshoot between 1.3 and 1.4-fold higher than in normoxia) ; return to normoxic consumption was observed after 4 and 7 hours in the smaller and larger *A. purpuratus* specimens, respectively. Oxygen debts in marine invertebrates occur to get rid of anaerobic end products, replenishing oxygen stores within body fluids and respiratory pigments and regenerating phosphogen and ATP stores depleted during hypoxia (Herreid, 1980). *M. edulis* larvae after anoxia and re-oxygenation exhibited an overshoot during the early aerobic recovery period (i.e. 30 min) followed by a gradual return to the steady state within 3 hours Wang and Widdows (1991). The adult mussels exhibited a similar recovery time but with a slow decrease of the oxygen-debt and, within the early aerobic recovery period, an energy production below the theoretical values for an aerobic metabolism (Shick et al., 1986). This suggests that *A. purpuratus* may use both metabolic depression and anaerobic metabolism below the P_{cO_2} , whatever the individual size.

The oxygen debt seems to be higher in the largest individuals when considering their higher standardized respiration rates and their longer recovery time compared with small scallops (Fig. 3.3). Bivalves *Scapharca inaequivalvis* and *Abra tenuis* activate their anaerobic metabolism with a evident metabolic depression, but rely mainly on aerobic metabolism even during severe hypoxia (Van den Thillart et al., 1992 ; Wang and Widdows, 1993). A higher use of anaerobic pathways instead of metabolic depression in large-sized individuals would lead to higher accumulation of anaerobic metabolism end products compared to the smaller ones, this hypothesis could be tested in a future research. A recovery in no more than 8.5 hours in this study may indicate that *A. purpuratus* the ability to cope with daily cyclic hypoxia crisis (that occur frequently in the Peruvian coast; Aguirre-Velarde et al., 2019b) and is able to regulate its oxygen consumption during and after these events. Chronic exposures to severe and prolonged hypoxic conditions affect *A. purpuratus* budget by reducing the energy available for the major metabolic functions affecting growth, reproduction and survival (Aguirre-Velarde et al., 2019a). Whereas previous studies dealing with this species focused on the small size classes (20-35 mm shell height) the present study reveals a lower ability of larger specimens. This result has to be considered at the time of evaluating the potential adaptation of the Peruvian scallop to main environmental stressors.

3.5 Conclusions

The results of this study emphasize size-regulated metabolic responses to progressive hypoxia for the Peruvian scallop, *A. purpuratus*. They will help to develop sustainable aquaculture strategies based on the location of the culture areas, the distance of the suspended culture devices from the seabed, the size of the individuals, and the duration of the upcoming hypoxic crisis. The high rates of oxygen consumption during the process of recovery from hypoxia suggest that a constant aeration of at least 5 hours is necessary after a long hypoxic event. In Peruvian bays, scallops may cope with daily cycles of temperature and oxygen saturation, but longer hypoxic episodes and chronic hypoxia may strongly affect scallop physiology and even survival. The identification of biochemical physiological proxies to understand mechanisms involved in a better tolerance to hypoxia for small animals in future studies, either in the laboratory or in the field, will help to deepen the knowledge on the acclimation and vulnerability of this species to climate change.

Chapter 4

Trade-off between growth and reproduction in *Argopecten purpuratus* (L.) scallops exposed to medium-term hypoxia and acidification

Journal of Aquaculture (submitted)

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Abstract

Peruvian bays that are home to aquatic organisms of commercial interest are increasingly affected by hypoxia and low pH events. These stressors produce unfavorable conditions for the aquatic fauna, leading to mass mortality events. In this study, we evaluated the ecophysiological responses of the scallop *Argopecten purpuratus* exposed to moderate hypoxia (20% DO saturation) and low pH (OA, pH 7.4) using a 2×2 factorial design. We found that a 51-day exposure to low pH (OA treatment) had greater negative effects on *A. purpuratus* performances than hypoxia (lower survival and decreased clearance and calcification rates), but stimulated early gonad maturation. The survival rate was 1.3-fold higher under hypoxia than under acidic treatment. The interaction between hypoxia and low pH had an antagonistic effect on survival, since the combined treatment (HOA) resulted in lower scallop mortality than the single stress treatments. Calcification was negatively affected by all stress treatments, whereas hypoxia and OA both stimulated gametogenesis. OA treatment resulted in higher frequencies of previtellogenic and vitellogenic oocytes, greater gonad coverage area, and lower frequency of atretic oocytes, suggesting higher reproductive potential. HOA was positively related to oocyte development and high frequency of post-vitellogenic and atretic oocytes. These results suggest that, due to hypoxia and low pH, feeding is reduced and energy allocation prioritizes scallop gonad maturation. This trend would have negative effects on scallop growth and calcification, while increased reproduction under environmental stress could mitigate the effect on recruitment.

Keywords : Hypoxia, low pH, homeostasis, gametogenesis, energy allocation.

4.1 Introduction

Climate change is affecting marine communities, reducing the exploitable stocks of fish and invertebrates (Pörtner et al., 2014). In most eastern boundary upwelling systems (EBUS), climate change could induce an intensification of oceanic upwelling, leading to regional cooling rather than warming of surface waters (Wang et al., 2015). This intensification can also promote seawater hypoxia and acidification, as well as biological productivity (Wang et al., 2015). Located in the South-Eastern Pacific, the Humboldt current system hosts one of the most productive ecosystems of the world (Chavez et al., 2008). It is subject to El Niño Southern Oscillation (ENSO) events, strong coastal waves, and a superficial Oxygen Minimum Zone (OMZ; Fréon et al., 2006; Graco et al., 2017). Environmental changes expected over the next few years could affect marine ecosystem production and related human activities (Barange et al., 2014).

The scallop *Argopecten purpuratus* is the most heavily fished and cultivated bivalve species in this coastal ecosystem. Recently, this species has been affected by environmental stressors such as hypoxia, sulfur events and harmful algal blooms, which have been identified as responsible for mass mortalities (Aguirre-Velarde et al., 2019; Kluger et al., 2019; Cueto-Vega et al., 2021). In northern Chile, the intensification of upwelling has led to high mortality of *A. purpuratus* populations, linked to low temperature, low dissolved oxygen (DO) and low pH (7.59 to 7.68 at 22 m depth; Ramajo et al., 2020). Other evidence of negative consequences of upwelling intensification on scallops was reported for the Peruvian coast: low temperature and hypoxia during summer induced lower growth and disruption of the reproductive cycle (Aguirre-Velarde et al., 2019). In Peru, the co-occurrence of low pH and hypoxia along the continental shelf is due to the presence of the shallow, intense and acidic Oxygen Minimum Zone (Friederich et al., 2008; Graco et al., 2017). In the central part of the Peruvian coast (Chimbote, 9°S), low pH (7.5) was measured close to 20 m depth, along with high $p\text{CO}_2$ (maximum 1,250 μatm ; Hernandez-Ayon et al., 2019).

Ecophysiological studies can help to understand the effect of hypoxia and low pH at the organism level, before extending it to the population level. The cumulative negative effects of low pH and dissolved oxygen (DO) appear to be very common in marine organisms; nevertheless, antagonistic effects have also been observed (Gobler et al., 2014). Experimental studies assessing the effects of these environmental stressors are still scarce. A study carried out by Ramajo et al. (2019) suggested that, after 11 days, juveniles prioritized calcification at the expense of somatic growth under upwelling conditions (14°C, pH 7.6 and 40% DO saturation). Hypoxia has recently been shown to be a chronic stress factor in shallow Peruvian bays: in summer, hypoxia may be present up to 20% of the time (Aguirre-Velarde et al., 2019; Cueto-Vega et al., 2021). Hypoxia can affect biological processes, such as rates of assimilation and mobilization of energy reserves in marine organisms (Thomas et al., 2019). Laboratory experiments on the effects of the sole hypoxia showed a decrease of scallop clearance and respiration rates (Aguirre-Velarde et al., 2016, 2018). Laboratory experiments on the mussel *Mytilus chilensis* exposed to low pH showed that medium-term exposure (pH close to 7.7 for 70 days) induced a significant reduction of clearance, absorption and oxygen consumption rates, resulting in a significant

drop of energy available for growth (*i.e.*, scope for growth; Navarro et al., 2013). Ramajo et al. (2016) found that, after 30 days of exposure to low pH (close to 7.6), *A. purpuratus* juveniles from Chile were able to cope with this stressor by enhancing its metabolic and calcification rates with higher food supply. In contrast, scallop juveniles from Peru showed a decrease in shell growth and calcification rates (by 9 and 10%, respectively) when facing low pH (7.4), while shell microhardness was positively affected (Córdova-Rodríguez et al., 2022).

A meta-analysis of 54 marine invertebrates showed that respiration rate and reproduction are the physiological processes most sensitive to hypoxia (Galic et al., 2019). *A. purpuratus* is a species with high fecundity and continuous gamete production throughout the year (Cantillanez et al., 2005). However, extreme environmental conditions linked to hypoxia can interrupt the species' reproductive cycle (Aguirre-Velarde et al., 2019) and reduce recruitment in the year following the event (Kluger et al., 2019). Although information on the effects of hypoxia and low pH on gonad maturation and reproduction is still scarce, it is known that mature and post-spawning scallops are more vulnerable to hypoxia and heat stress than immature scallops (Brokordt et al., 2015; Salgado-García, 2023).

Responses to the combined action of hypoxia and acidification in *A. purpuratus* have not yet been studied over a long period. Therefore, the objectives of the present study were: 1) to compare the ecophysiological responses of *A. purpuratus* to single and combined stress factors (hypoxia and low pH), 2) to identify changes over time and 3) to investigate the effects on gametogenesis and reproductive potential of survivors. The results of this work could help to understand the capacity and/or vulnerability of this exploited resource to the increasing and abrupt changes that occur during extreme events in the Humboldt Current system.

4.2 Material and Methods

Juvenile scallops ($n = 1500$, 3-month age, 12.7 ± 1.6 mm SD shell height) were provided by the hatchery AgroMar del Pacífico (Piura, Peru) and transferred in a cool box to the Aquatic Ecophysiology Laboratory of IMARPE (Callao, Peru). Once in the laboratory, scallops were distributed into eight 64-L tanks continuously supplied in filtered ($10 \mu\text{m}$), thermoregulated (ca. 16.5°C) and oxygen-saturated (100% DO) seawater (7.3 L h^{-1}). After an acclimation period of one week, scallops of similar size were selected and 90 specimens were randomly placed into 12 rectangular 44-L tanks.

4.2.1. Experimental design and methods

A 2×2 factorial experiment (two oxygen levels: hypoxia and normoxia, and two pH levels: low and normal pH conditions) was carried out over a period of 51 days. The four treatments were hypoxia only (HYPO: hypoxia/non-OA), acidification only (OA: normoxia/OA), both stresses combined (HOA: hypoxia/OA), and control (CTRL: normoxia/non-OA). Hypoxia consisted in

exposing the scallops to an oxygen saturation of 20%, which is close to their oxygen critical point, P_{cO_2} (range 14.7-24.4%). When oxygen saturation falls below this threshold, scallops become oxy-conformers rather than oxy-regulators (Aguirre-Velarde et al., 2016). The OA condition was set up at pH 7.4, as this low pH value was found for several days in conditions favorable to upwelling in other coastal waters influenced by the Humboldt system (Arauco Gulf, central Chile; Saavedra et al., 2021). Furthermore, a previous study reported the negative effects of such a high level of acidification on the growth and calcification of *A. purpuratus* shells (Córdova-Rodríguez et al., 2022). The DO and pH values of the control condition (ca. 100% DO saturation and pH 7.8) were consistent with values typically found in Peruvian bays (Carhuapoma, pers. comm.), especially during the Austral summer.

Oxygen saturation in hypoxic treatments (HOA and HYPO) was automatically controlled in the experimental tanks by gaseous nitrogen injection into the seawater, using the system described in Artigaud et al. (2014). Acidification was achieved by controlled bubbling of CO_2 using a pH controller (Tunze 7070/2, Germany) connected to a CO_2 cylinder which released the suitable amount of gas into a 400-L collection tank. From this tank, the filtered seawater (UV and 10- μ m) flowed continuously (5.3 L h⁻¹) into the six aquaria with acidic conditions. In normal pH condition (7.8), seawater was filtered (UV and 10- μ m) and flowed directly into the tanks at the same rate as previously mentioned.

In all aquariums, seawater was thermoregulated (16.5 °C) and homogenized using pumps. To prevent gas exchange with the atmosphere, the water surface in all aquariums was covered with floating polystyrene sheets. Oxygen saturation, temperature and pH were measured daily in each aquarium using a multi-parameter probe (WTW multi 3430) connected with an optical oxygen sensor (FDO 925-3 dissolved oxygen probe, WTW, USA) and a pH electrode (Sentix® 940-3). Electrodes were calibrated daily on the NBS scale for pH (pH 4 and 7). Scallops in each aquarium were fed continuously with an algal mixture (*Isochrysis galbana* and *Chaetoceros calcitrans* 1:1) using peristaltic pumps to maintain a concentration above 9000 cells mL⁻¹. All tanks were cleaned daily of bio-deposits using a siphon to remove the feces; in addition, dead scallops were removed from the tanks. Due to mortality in some treatments and to avoid a density effect between treatments, some scallops were removed from the tanks after 30 days in order to keep 50 specimens per tank. At the beginning of the experiment, and then bi-weekly during 51 days, 10 scallops were randomly sampled from each aquarium. Individual shell height (H, mm) was measured using a caliper (to the nearest 0.01 mm). Then, shell and excised soft tissues (gonad, muscle, mantle and remaining tissues) were wet-weighed, dried for 96 h in an oven at 60 °C and dry-weighed (to the nearest 0.001 g). At the end of the experiment (day 51), three whole gonads from specimens in each aquarium (nine per treatment, 36 gonads in total) were wet-weighed, fixed in Davidson's solution for 48 h (Kim et al., 2006) and preserved in 70% ethanol at 4 °C until processed for histological analysis.

4.2.2. Carbonate system parameter measurements

During the experimental period, total alkalinity (TA) was monitored once a week to estimate carbonate system parameters in 100 mL water samples taken from each aquarium. TA was measured using a titration system (775 Dosimat, Metrohm Ltd., Herisau, Switzerland). Measured temperature, pH_{NBS} , TA and salinity were used to calculate other carbonate system parameters and calcite (Ω_{cal}) and aragonite (Ω_{ara}) saturations. Analyses were performed using CO₂SYS for MS Excel (Pierrot et al., 2006) with Mehrbach solubility constants (Mehrbach et al., 1973) adjusted by Dickson and Millero (1987), and KHSO₄ (Dickson, 1990). Average carbonate system parameters are reported in Table 4.1.

4.2.3. Survival and physiological rates

Survival rate was calculated as the daily percentage of survivors; from day 31 on, survival rate was adjusted to the new aquarium density mentioned above.

Short-term (14 days) and medium-term (51 days) responses to treatments were assessed by estimating the following descriptors on both time periods: survival, clearance rate, growth rate, condition index, net calcification and gonadosomatic index. Clearance rate measurements were made using a fluorometer (AquaFlash™, Turner designs, USA) to quantify chlorophyll-a concentrations (arbitrary units) in the water outflow of each aquarium. Fluorescence data obtained during clearance rate measurements were first corrected using the fluorometer baseline (determined during fluorometer calibration).

Individual clearance rate (CR , L h⁻¹ ind⁻¹; Hildreth and Crisp, 1976) was calculated according to the formula:

$$CR = \frac{F_R (Fluo_{in} - Fluo_{out})}{Fluo_{in} \times n_{ind}}$$

where, F_R is the water flow rate through the aquarium (L h⁻¹) and n_{ind} is the number of individuals into the aquarium. $Fluo_{in}$ and $Fluo_{out}$ are the chlorophyll-a concentrations measured in the water inflow and outflow of the aquarium. In order to standardize clearance rates from potential variations in mean individual size between aquariums, clearance rates were standardized to a 30-mm-shell-height individual using Bayne et al. (1987) formula:

$$CR_{std} = CR \left(\frac{H_{std}}{H_{mean}} \right)^b$$

where, CR_{std} is the standardized clearance rate for an individual of shell height H_{std} (30 mm), CR is the estimated clearance rate, H_{mean} is the mean scallop shell height in the aquarium, and b is the allometric coefficient relating size and clearance rate. This coefficient was taken equal to 2 (Pouvreau et al., 1999 recalculated for height by Aguirre et. al., 2018).

Shell growth rates (GR, mm d⁻¹) at day t were calculated per aquarium as the difference in mean shell height between day t and the starting day (t_0) divided by the number of days elapsed.

The Fulton's condition index (CI, g cm⁻³; Ricker, 1975) was calculated for the dried soft tissues according to the formula:

$$CI = \frac{FDW}{H^3} \times 1000$$

where, FDW is the flesh dry weight (g) and H is the shell height (mm) of the individuals.

Net calcification rates (NCR, mg CaCO₃ g⁻¹ d⁻¹) were calculated as the change in shell DW between t and t_0 divided by the number of days elapsed and normalized to the initial shell DW of the individuals.

The amount of calcium carbonate (DW.CaCO₃, mg) was calculated using the mean shell dry weight (SDW, g) and the formula of Córdova-Rodríguez et al. (2022) for *A. purpuratus* juveniles:

$$DW.CaCO_3 = 0.1995 + 0.9950 SDW$$

The Gonadosomatic index (GI, %) was calculated as the ratio between the gonad DW and total soft tissue DW multiplied by 100 (Sastry, 1970).

4.2.4. Quantitative histology

Stored gonads were dehydrated in progressively increasing ethanol concentration (70%, 80%, 95%, and 100%), cleared in xylene and embedded in paraffin (Paraplast Plus, Leica Biosystems, Richmond, IL, USA). Slides were prepared in 4-μm thick sections using a rotary microtome (Leica RM 2155, Leica Microsystems), dried overnight at 37 °C, and mounted on glass slides. Sections were deparaffinized and rehydrated in regressive series of ethanol (Shandon Citadel 2000 Tissue Processor) before staining. Each slide was then stained for 5 min with Harris's hematoxylin and counterstained for 12 min with eosin phloxine (Kim et al., 2006). Cover slips were applied with synthetic mounting media. Finished slides were observed under a light inverted-microscope (Leica DMIRB) at ×20 magnification to characterize scallop gonad development and reproductive potential.

Gonadal development was assessed according to the frequency of oocytes and hemocytes in the ovary, by counting the total number of each cell type appearing in five randomly selected images (×20 magnification; 1.44 mm²) of the ovary digitized with a Retiga 2000R (Qimaging®) digital camera. Reproductive potential was assessed measuring the gonad coverage area (GCA), gamete development index (GDI) and oocyte morphometric quality on five regions of the ovary (García-Corona et al., 2018; Rodríguez-Jaramillo et al., 2022). Oocytes were classified according to their development stage as oogonia, previtellogenic, vitellogenic, post-vitellogenic and atretic (Mazón-Suástegui et al., 2021). The surface area occupied by the gonad was determined from the mean of five images from each specimen processed with the Image Pro Plus 5.1 software

(Media Cybernetics, Bethesda, MD, USA). Image analysis was based on the intensity of gonad tissue-specific color; germinal tissue coverage was automatically calculated by the software and the surface area was reported as the gonad coverage area (GCA %) according to García-Corona et al. (2018) modified from Rodríguez-Jaramillo et al. (2008) as:

$$GCA = \frac{GA}{TA} \times 100$$

where GA is the gonad occupation area (μm^2) and TA is the total area (μm^2). The gamete development index (GDI) was calculated as the ratio of post-vitellogenic oocytes to the total number of oocytes present in the gonad, excluding atretic and reabsorbed oocytes (Rodríguez-Jaramillo et al., 2017).

In order to determine the morphometric quality of late-developed oocytes (vitellogenic and post-vitellogenic), 20-30 oocytes per type were measured from 5 images at $\times 40$ (2.88 mm^2) using the software Image Pro Plus 5.1. Then, the oocytes were manually measured to obtain the oocyte surface area (μm^2). To minimize size variations in growing oocytes and standardize their shape to a sphere, the theoretical diameter (TD, μm ; Saout et al., 1999) was calculated from the total area (A, μm^2) of each oocyte using the following formula:

$$TD = \sqrt{\frac{4A}{\pi}}$$

An estimate of the amount of ovoplasm in the oocytes was calculated according to García-Corona et al. (2018). For this purpose, the area of the nucleus was determined for each oocyte similarly to the total oocyte area using digitized images at $\times 20$. The nucleus/cytoplasm ratio (N/C) was calculated as:

$$N/C = \frac{NTA}{CTA} \times 100$$

Where NTA is the nucleus total area and CTA is the cytoplasm total area.

The shape index (SI) of the oocytes was automatically calculated as the percentage of deviation from a circular shape (García-Corona et al., 2019) by processing the images at $\times 40$ using SigmaScan (Systat Software Inc., San Jose, CA, USA).

4.2.5. Data analyses

All analyses were performed using R 3.6.4 software (R Core Team, 2021). Prior to the statistical analyses, the assumptions of normality and homogeneity of variance were verified using the Shapiro-Wilk and Levene's tests, respectively. To compare survival curves between treatments, we performed Wald test along with Cox proportional hazard model from "survival" and "survminer" R-packages (Therneau and Grambsch, 2013). Differences in survival percentages and physiological rates (clearance rate, growth rate, condition index, net calcification rate and gonadosomatic index) were tested using a two-way factorial ANOVA after 14 and 51 days of stress exposure, setting pH and oxygen as factors.

Indicators of gonadal development (oocyte and hemocyte frequencies in the ovary) and reproductive potential (gonadal coverage area, gamete development

index and morphometric features illustrating oocyte quality) were tested using a separate one-way ANOVA followed by a Tukey's HSD post hoc test for comparison of means. Prior to analyses, values expressed in percentages (gonadal coverage area, gamete development index and shape index) were arcsine ($\arcsine \sqrt{P}$) transformed.

A principal component analysis (PCA) was computed to better understand the relationships between the physiological indicators mentioned above and the stress treatments. All variables were auto-scaled to zero before PCA using the “FactoMineR” R package (Lê et al., 2017).

Table 4.1. Mean (\pm SE) dissolved oxygen saturation (DO sat), pH_{NBS} , temperature, pCO_2 , total alkalinity (TA), and calcite ($\Omega_{calcite}$) and aragonite ($\Omega_{aragonite}$) saturation states for *Argopecten purpuratus* low pH - hypoxia experiment. Temperature and salinity were stable at 16°C and 34, respectively. Estimated parameters were calculated using CO2SYS software.

Parameters	HYPO	OA	HOA	CTRL
<i>Measured</i>				
Oxygen (% of saturation)	19.77 \pm 0.40	98.16 \pm 0.40	19.93 \pm 0.46	99.31 \pm 0.46
pH_{NBS}	7.63 \pm 0.03	7.39 \pm 0.02	7.34 \pm 0.02	7.76 \pm 0.02
Temperature (°C)	16.93 \pm 0.08	16.88 \pm 0.07	16.89 \pm 0.02	16.97 \pm 0.09
Total alkalinity (μ mol/kg)	2109.5 \pm 12.36	1996.6 \pm 13.60	2120.2 \pm 6.74	1992.6 \pm 15.29
<i>Calculated</i>				
pCO_2 (μ atm)	1200.0 \pm 119.5	2056.0 \pm 50.2	3312.0 \pm 139	942.0 \pm 8.13
calcite ($\Omega_{calcite}$)	1.18 \pm 0.10	0.59 \pm 0.10	0.49 \pm 0.02	1.2 \pm 0.25
aragonite ($\Omega_{aragonite}$)	0.82 \pm 0.07	0.41 \pm 0.01	0.34 \pm 0.02	0.83 \pm 0.02

4.3 Results

4.3.1. Survival

Significant differences ($p < 0.0001$, Wald test) were observed in survival rates between control and stress treatments. HOA, HYPO and OA treatments increased mortality risk by a factor of 2.27 ($p < 0.001$, Wald test), 2.57 ($p < 0.0001$) and 3.29 ($p < 0.0001$), respectively. After 14 days, survival rate in all treatments was higher than 90%. Values were lower after 51 days: the OA treatment exhibited the lowest survival rate (46.2% \pm 5.7, SE), followed by HYPO (62.2% \pm 13.5), HOA (70.3% \pm 0.9) and CTRL (88.0% \pm 4.4; see Table 4.2 and

Fig. 4.1A). This trend was also demonstrated by significant interaction between oxygen and pH on survival rate for short- (14 days) and long-term (51 days) exposures (two-way factorial ANOVA, $p < 0.05$; Table 4.3).

4.3.2. Physiological measurements after 14 and 51 days

After 14 days of experiment, standardized clearance rate (SCR), growth rate (GR), net calcification rate (NCR) and condition index (CI) were significantly negatively affected by low pH (two-way factorial ANOVA, $p < 0.05$; Table 4.3). Hypoxia only significantly affected SCR and NCR. SCR at day 14 was lower in the stress treatments (negative effect of acidification and/or hypoxia, Table 4.2) than in the control; the SCR values for HYPO, OA and HOA represented 65, 56 and 47 % of the value for CTRL, respectively. NCR was also lower in the stress treatments than in the control; the HYPO condition had a NCR value 30% lower than that of the CTRL, whereas the OA and HOA treatments had values 59% and 78% lower than the one for CTRL, respectively. GR was lower in acidified treatments (0.21 and 0.17 mm d⁻¹ in OA and HOA, respectively) than in normal pH treatments (0.26 and 0.29 mm d⁻¹ in HYPO and CTRL, respectively). Conversely, there was a significant positive effect of acidification on the condition index (two-way factorial ANOVA, $p < 0.05$): CI was higher in acidified treatments (0.013-0.014 g cm⁻³) than in normal pH treatments (0.011 g cm⁻³; Table 4.2). Differences between treatments were not significant at day 14 for the Gonadosomatic index (GI).

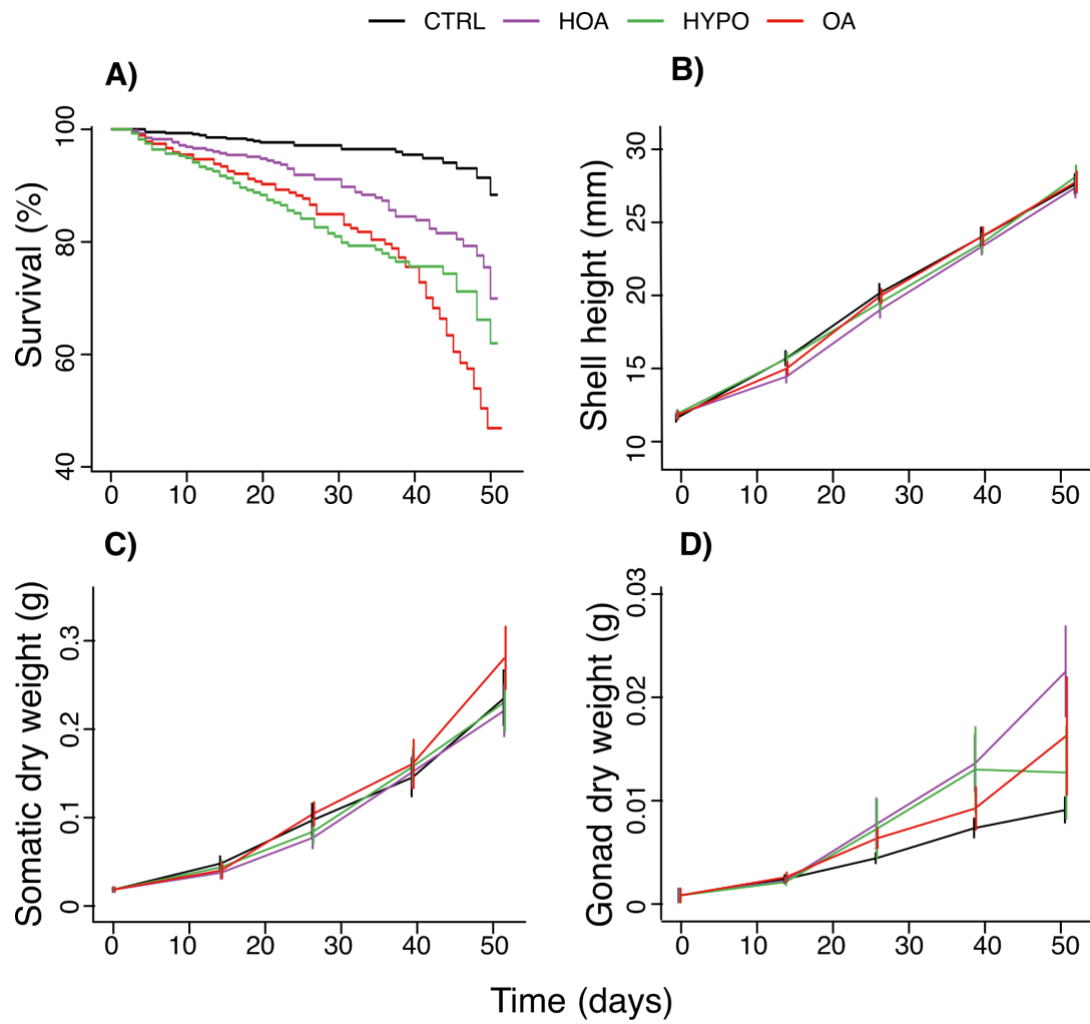


FIG. 4.1. Survival rate (A), growth in shell height (B), and somatic (C) and gonad dry weight (D) along the 51-day experiment performed at IMARPE. Experimental treatments: single hypoxia (HYPO), single acidification (OA), combined hypoxia and acidification (HOA) and control (CTRL). Bars indicate 95% confidence intervals.

Table 4.2: Summary of survival and physiological rates in *A. purpuratus* after 14 and 51 days under single hypoxia (HYPO), single acidification (OA), combined hypoxia and acidification (HOA), and control (CTRL). Values are means \pm SE. Bold numbers indicate significant differences at $p < 0.05$ (1-way ANOVA).

Physiological rates	HYPO	OA	HOA	CTRL	<i>p</i>
<i>After 14 days</i>					
Survival (%)	85.6 \pm 4.5	90.4 \pm 1.3	92.6 \pm 1.9	96.3 \pm 0.7	0.089
Standardized clearance rate (L.h ⁻¹ .ind ⁻¹)	2.2 \pm 0.3	1.9 \pm 0.1	1.6 \pm 0.2	3.4 \pm 0.4	0.004
Growth rate (mm d ⁻¹)	0.26 \pm 0.01	0.21 \pm 0.03	0.17 \pm 0.01	0.29 \pm 0.03	0.023
Condition index (g cm ⁻³)	0.011 \pm 0.00	0.013 \pm 0.00	0.014 \pm 0.001	0.011 \pm 0.000	0.008
Net calcification rate (mg CaCO ₃ d ⁻¹ g ⁻¹)	64.6 \pm 6.7	37.7 \pm 8.6	20.4 \pm 2.8	92.6 \pm 11.4	0.001
Gonadosomatic index (%)	4.3 \pm 0.1	6.1 \pm 1.0	5.6 \pm 0.3	4.9 \pm 0.3	0.195
<i>After 51 days</i>					
Survival (%)	62.2 \pm 13.5	46.2 \pm 5.7	70.3 \pm 0.9	88.0 \pm 4.4	0.028
Standardized clearance rate (L.h ⁻¹ .ind ⁻¹)	2.8 \pm 0.2	1.1 \pm 0.2	1.5 \pm 0.2	2.6 \pm 0.2	0.001
Growth rate (mm d ⁻¹)	0.30 \pm 0.01	0.30 \pm 0.01	0.29 \pm 0.01	0.31 \pm 0.00	0.252
Condition index (g cm ⁻³)	0.011 \pm 0.001	0.014 \pm 0.001	0.012 \pm 0.000	0.011 \pm 0.001	0.220
Net calcification rate (mg CaCO ₃ d ⁻¹ g ⁻¹)	156.1 \pm 1.1	155.4 \pm 6.9	128.6 \pm 12.4	180.3 \pm 13.6	0.038
Gonadosomatic index (%)	8.1 \pm 1.8	8.1 \pm 1.4	12.1 \pm 0.7	4.9 \pm 0.1	0.018

After 51 days, SCR, NCR and GI were significantly affected by acidification (two-way factorial ANOVA, $p < 0.05$; Table 4.3), contrary to GR and CI. Scallops in the acidified treatments showed clearance rates at least 42% lower than those of individuals exposed to normal pH (1.1 and 1.5 L.h⁻¹.ind⁻¹ in OA and HOA vs. 2.8 and 2.6 L.h⁻¹.ind⁻¹ in HYPO and CTRL, respectively; Table 4.2). The highest Net Calcification Rate was found in the CTRL treatment, followed by HYPO, OA

and HOA, with mean values of 180.3, 156.1, 155.4 and 128.6 mg CaCO₃ g⁻¹ d⁻¹, respectively. Thus, the combination of hypoxia and acidification had a greater impact on shell calcification than the single stress treatments (similar NCR values). Overall, a 28.6% decrease in net calcification rate was observed between CTRL and HOA. Dead or dying specimens in the acidified treatments also showed weakness or rupture of the hinge ligament throughout the experiment. About 30% of the dead scallops showed this symptom, overall. In addition, we visually observed (no quantification) a greater abundance of biodeposits in the aquariums with stress treatments, compared to control treatment. Acidification and hypoxia positively affected gonadosomatic index after 51 days: scallops exposed to the combined stress treatment (HOA) showed the highest GI (12.1 %), followed by OA (8.1 %), HYPO (8.1 %) and CTRL (4.9 %; Table 4.2).

Table 4.3. Effect of oxygen and pH treatments on survival and physiological rates in *A. purpuratus* after 14 and 51 days of experiment. Two-way factorial ANOVA; the *F*-test statistic, sum of squares (SS) and degrees of freedom (*df*) are reported. Bold numbers indicate significant differences at $p < 0.05$.

Response	Source	<i>df</i>	SS	<i>F</i>	<i>p</i>
<i>After 14 days</i>					
Survival (%)	Oxygen	1	54.42	2.79 9	0.1 33
	pH	1	0.93	0.04 8	0.8 33
	Oxygen x pH	1	126.03	6.48 1	0.0 34
	Error	8	155.56		
Standardized clearance rate (L.h ⁻¹ .ind ⁻¹)	Oxygen	1	1.95	9.66 8	0.0 14
	pH	1	3.48	17.2 56	0.0 03
	Oxygen x pH	1	0.584	2.89 3	0.1 27
	Error	8	1.613		
Growth rate (mm d ⁻¹)	Oxygen	1	0.0026	1.71 5	0.2 27
	pH	1	0.0224	15.0 2	0.0 05
	Oxygen x pH	1	0.0001	0.09	0.7 72
	Error	8	0.0119		
Condition index (g cm ⁻³)	Oxygen	1	3.20E- 07	1	0.4 68
	pH	1	1.26E- 05	23	0.0 01
	Oxygen x pH	1	4.64E- 07	1	0.3 86
	Error	8	4.41E- 06		
Net calcification rate (mg CaCO ₃ d ⁻¹ g ⁻¹)	Oxygen	1	1523	7.82 2	0.0 23
	pH	1	7362	37.8 2	0.0 00
	Oxygen x pH	1	79	0.40 4	0.5 43
	Error	8	1557		

Gonadosomatic index (%)	Oxygen	1	0.809	0.875	0.377
	pH	1	4.683	5.068	0.055
	Oxygen x pH	1	0	0.001	0.982
	Error	8	7.394		
After 51 days					
Survival (%)	Oxygen	1	2.3	0.013	0.911
	pH	1	855.9	4.869	0.058
	Oxygen x pH	1	1866.8	10.619	0.012
	Error	8	1406.3		
Standardized clearance rate (L.h ⁻¹ .ind ⁻¹)	Oxygen	1	0.075	0.708	0.428
	pH	1	5.147	48.484	0.000
	Oxygen x pH	1	0.056	0.524	0.493
	Error	7	0.743		
Growth rate (mm d ⁻¹)	Oxygen	1	0.0001	0.409	0.540
	pH	1	0.0005	4.295	0.072
	Oxygen x pH	1	0	0.278	0.612
	Error	8	0.001		
Condition index (g cm ⁻³)	Oxygen	1	1.52E-07	0	0.793
	pH	1	5.22E-06	3	0.151
	Oxygen x pH	1	5.98E-06	3	0.127
	Error	8	1.65E-05		
Net calcification rate (mg CaCO ₃ d ⁻¹ g ⁻¹)	Oxygen	1	2105.6	7.179	0.028
	pH	1	1906.9	6.501	0.034
	Oxygen x	1	0.2	0.00	0.9

	pH			1	81
	Error	8	2346.4		
Gonadosomatic index (%)	Oxygen	1	38.16	8.98 3	0.0 17
	pH	1	39.47	9.29 2	0.0 16
	Oxygen x pH	1	0.53	0.12 4	0.7 34
	Error	8	33.98		

4.3.3. Gonad maturation and reproductive potential

Histological observations of gametogenesis indicated no ripening of the testicular portion of the gonads in the CTRL condition, evidenced by abundant connective tissue and clutches of spermatogonia attached to the walls of the testicular acini (Fig. 4.2A). A strong hemocyte infiltration was also observed in this condition. No apparent differences in spermatogenesis were observed between the HYPO, OA and HOA treatments (Fig. 4.2B, C and D). For these three conditions, acini were surrounded by scarce connective tissue and full of spermatids that gave rise to the spermatozoa in a centripetal pattern towards the lumen of testicle ducts. Similar to the testicular portion of the gonads, ovary maturation was not observed in scallops from the CTRL group (Fig. 4.2E). In the latter, patches of gonial cells could be found particularly near the gonad ducts. The ovarian follicles were surrounded by abundant connective tissue and large quantities of hemocytes reabsorbing the maturation residues were observed in the gonad. The scallops in HYPO condition were in late vitellogenesis (Fig. 4.2F). The follicles were large and filled with pedunculated vitellogenic oocytes attached to the epithelium. Nonetheless, numerous atretic oocytes were located in the lumen of the follicles, together with few oogonia and previtellogenic oocytes. In the OA condition, ovaries were mainly in early vitellogenesis: small follicles interconnected to the gonad ducts were surrounded by abundant connective tissue and circulating hemocytes (Fig. 4.2G). Patches of oogonia could be seen on the walls of the follicles. Previtellogenic oocytes had scarce acidophilic cytoplasm; some vitellogenic and post-vitellogenic oocytes were present. Finally, scallops exposed to the HOA condition showed advanced ovarian ripeness. The follicles were large and filled with round-polygonal postvitellogenic oocytes or pedunculated vitellogenic oocytes, both with copious amounts of cytoplasm. Clutches of oogonia and some previtellogenic oocytes could still be found attached to the follicle epithelium, while the connective tissue was reduced (Fig. 4.2H). The frequency of each type of oocytes in female gonads is shown in Fig. 4.3A. The number of oogonias was significantly highest (one-way ANOVA; $p < 0.05$) in scallops from the CTRL (135 ± 14.3 cells. area⁻¹), followed by those exposed to the HYPO and OA treatments (ca. 78 cells. area⁻¹), while the lowest amounts of oogonias (29 ± 2.9 cells. area⁻¹) were registered in scallops exposed to the HOA condition. No difference (ca. 31 cells. area⁻¹; one-way ANOVA; $p > 0.05$) was found in the frequencies of previtellogenic oocytes between treatments. On the other hand, the number of vitellogenic oocytes was significantly higher (one-way ANOVA; $p < 0.05$) in treatment OA (51 ± 3.7 cells. area⁻¹), followed by treatments HOA (30 ± 3.2 cells. area⁻¹), HYPO and CTRL (17 ± 4.9 and 12 ± 6.4 cells. area⁻¹, respectively). Significantly higher frequency of post-vitellogenic oocytes occurred in scallops from treatments OA, HYPO and HOA, with ca. 32 cells. area⁻¹ vs. 1 ± 0.4 cells. area⁻¹ in the CTRL group. Atretic oocytes were significantly more abundant (one-way ANOVA; $p < 0.05$) in the ovaries of scallops placed in hypoxic conditions (HYPO: 16 ± 6.8 cells. area⁻¹, HOA: 18 ± 3.3 cells. area⁻¹), compared to OA (8 ± 3.3 cells. area⁻¹) and CTRL

(no atretic oocytes). Hemocyte infiltration significantly peaked (one-way ANOVA; $p < 0.05$) in the ovaries of scallops from the CTRL treatment (101 ± 9.8 cells. area⁻¹; more details in Table 4.4). Meanwhile, the abundance of circulating hemocytes was lower and not statistically different between the stress treatments (mean of ca. 23 cells. area⁻¹, $p > 0.05$; Fig. 4.3A).

Scallop reproductive potential was estimated from the gonad coverage area (GCA) and the gamete development index (GDI) in ovarian tissue. GCA was higher in scallops of the stress treatments (with values above 18.3%, $p < 0.05$; Fig. 4.3B) compared to CTRL individuals ($9 \pm 1\%$). The highest GDI was found for scallops kept in HOA condition ($32 \pm 3.7\%$), followed by those kept in HYPO ($20 \pm 5.3\%$) and OA ($16 \pm 3.2\%$) treatments. Significantly lower GDI ($0 \pm 0.1\%$; $p < 0.05$), and therefore lowest reproductive potential, was found in specimens from the CTRL. Additionally, gamete quality was evaluated by measuring mean morphometric features of late-developing oocytes (vitellogenic and post-vitellogenic), i.e. those with the greatest potential to contribute to offspring (Table 4.4). No significant difference between treatments ($p < 0.05$) was found in the theoretical diameter (TD), cytoplasm quantity (N/C) and shape index (SI) of vitellogenic oocytes. By contrast, significantly lower TD and SI values were recorded in postvitellogenic oocytes from scallops exposed only to OA.

Table 4.4. Mean morphometric features (TD: theoretical diameter; N/C: nucleus/cytoplasm ratio; SI: shape index) in late-developed oocytes in *A. purpuratus* exposed to single hypoxia (HYPO), single acidification (OA), combined hypoxia and acidification (HOA), and control (CTRL) for 51 days. The measurement of the morphometric features of the oocytes in the control group was not possible due to scallop gonad immaturity. Data (mean \pm SE) were analyzed using the treatments (three levels) as independent variables in univariate ANOVAs. The *F*-test statistic and degrees of freedom (*df*) are reported. Bold numbers indicate statistically significant differences at $p < 0.05$.

Oocyte type/Treatment	HYPO	OA	HOA	<i>F</i> (<i>df</i> = 2,3)	<i>p</i>
<i>Vitellogenic</i>					
TD (μ m)	33.7 ± 0.4	32.9 ± 0.4	34.0 ± 0.4	2	0.136
N/C (%)	41.4 ± 1	38.8 ± 1.1	38.7 ± 1.1	2	0.132
SI (%)	88.2 ± 0.3	88.0 ± 0.3	88.7 ± 0.2	2.3	0.103
<i>Post-vitellogenic</i>					
TD (μ m)	37.7 ± 0.4	36.4 ± 0.3	37.0 ± 0.3	4.6	0.010
N/C (%)	40.2 ± 1.1	39.3 ± 0.9	40.7 ± 1.3	0.4	0.666
SI (%)	88.5 ± 0.3	88.0 ± 0.3	88.9 ± 0.2	3.7	0.02

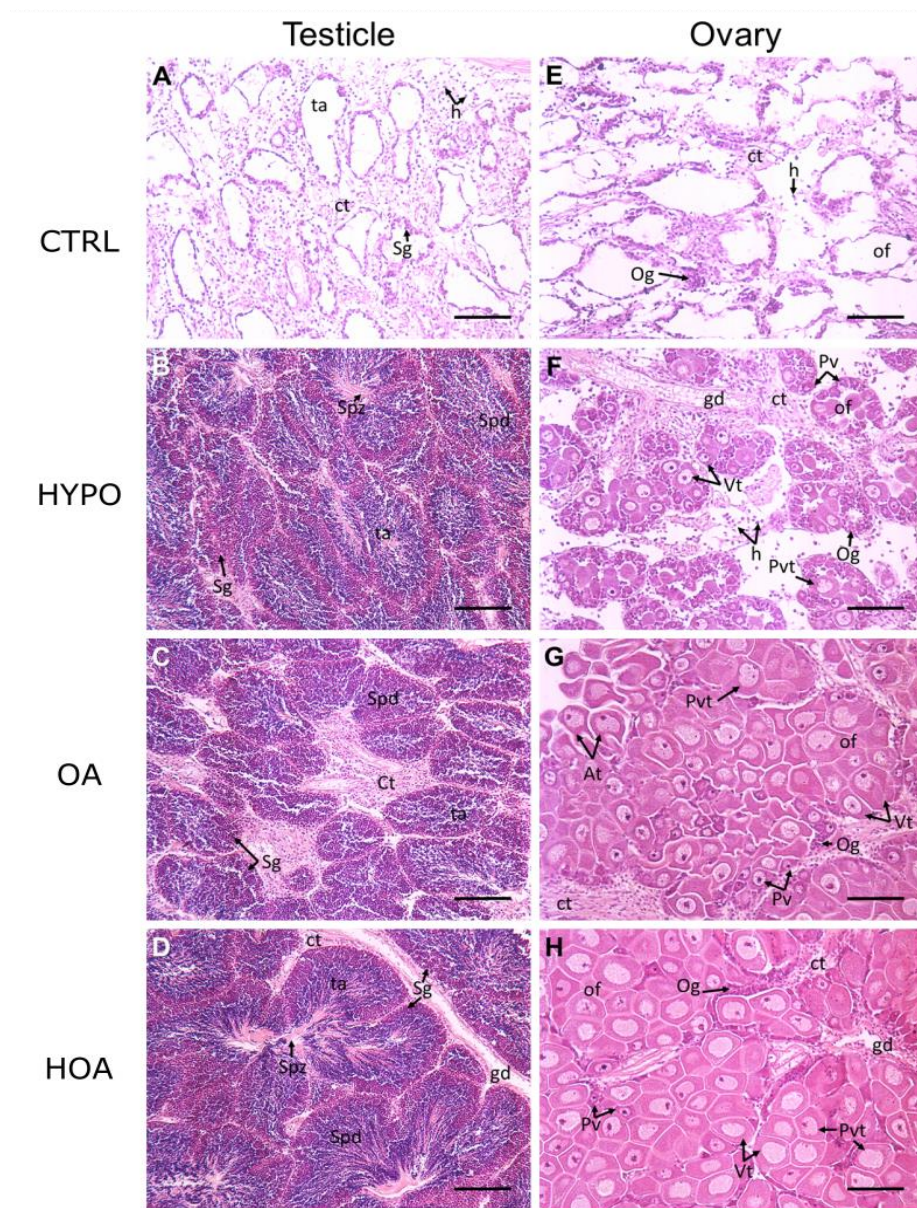


Fig. 4.2. Gonad development of *Argopecten purpuratus* specimens exposed to single hypoxia (HYPO), single acidification (OA), combined hypoxia and acidification (HOA), and control (CTRL) treatments, during 51 days. Sections were stained with Haematoxylin-eosin. ct: connective tissue, fl: ovarian follicles, gd: gonad ducts, h: haemocytes, Pvt: post-vitellogenic oocytes, Og: oogonia, Pv: previtellogenic oocytes, At: atretic oocytes, Vt: vitellogenic oocytes, ta: testicular acini, Sg: spermatogonia, Spd: spermatid, Spz: spermatozoa. Scale bar: 50 μ m.

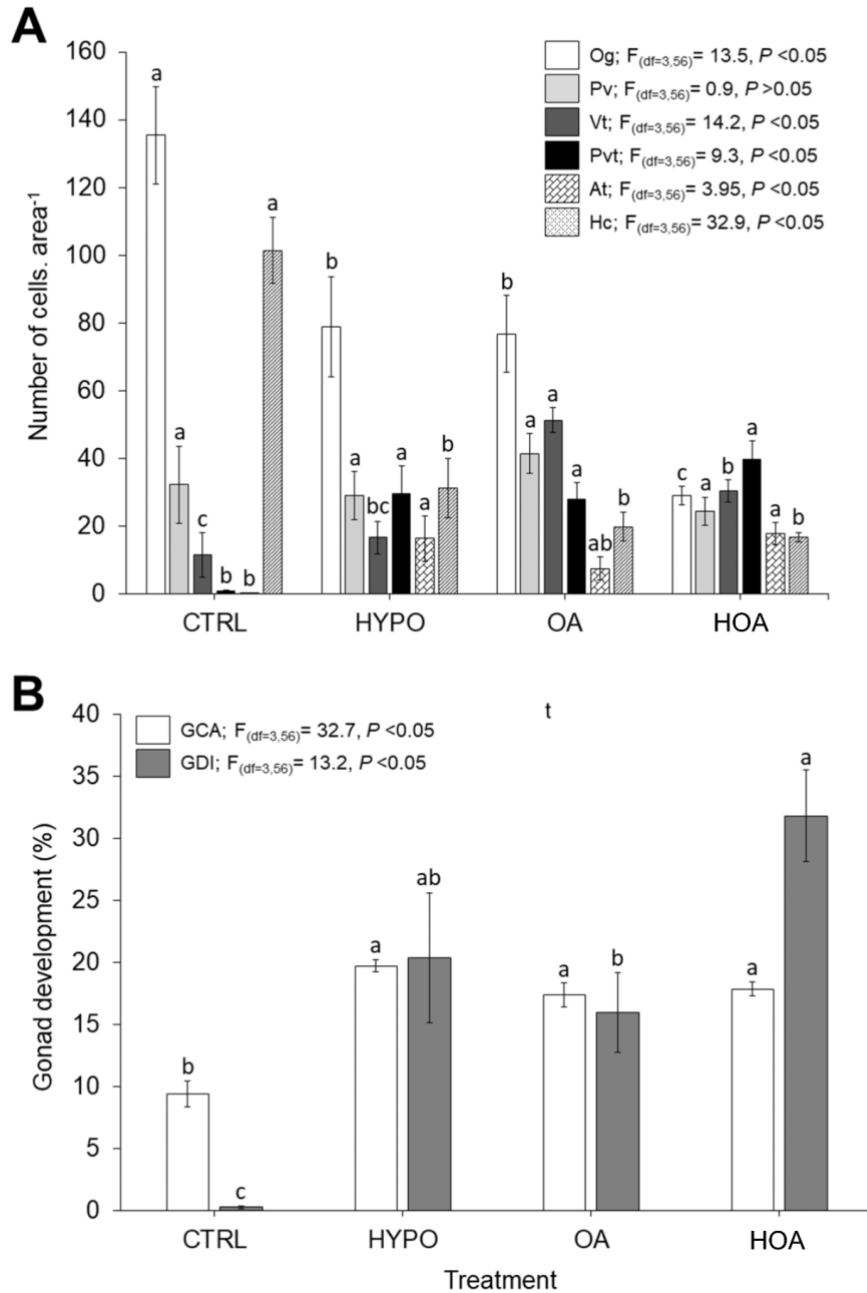


FIG. 4.3. Reproductive potential of *A. purpuratus* exposed to single hypoxia (HYPO), single acidification (OA), combined hypoxia and acidification (HOA), and control (CTRL) treatments, during 51 days. (A) Mean numbers of oocytes and hemocytes per female (at $\times 20$); (B) Reproductive potential (Gonad Coverage Area = GCA; Gamete Development index = GDI). Cell types are distributed in Oo: oogonia; Pv: previtellogenic oocytes; Vt: vitellogenic oocytes; Pvt: post-vitellogenic oocytes; At: atretic oocytes; Hc: hemocytes. Data (mean \pm SE) were analyzed using the treatments (four levels) as independent variables in one-way ANOVA for each cell type, GCA and GDI. The F-test statistic and degrees of freedom (df) are reported. Different superscript letters denote statistically significant differences at $p < 0.05$.

4.3.4. Relationships between physiological descriptors

The first and second principal components of the PCAs contributed 77.5% of the variability between physiological descriptors and 79.8% of the overall variability in scallop histological data at the end of the experiment (Fig. 4.4). In each PCA, a clear difference was found between treatments with acidic conditions (OA and HOA) and CTRL, whereas it was not possible to find a grouping in the HYPO treatment, due to its high variability. After analyzing the ecophysiological descriptors, the OA treatment was positively related with high condition index, but had lower values in clearance and survival rates. The HOA treatment was positively related with high gonadosomatic index, but exhibited lower clearance and growth rates. With regard to descriptors of reproductive potential, OA was associated with higher frequencies of previtellogenic and vitellogenic oocytes, high gonad coverage area (GCA), and low frequency of atretic oocytes. HOA was positively related with high gamete development index (GDI), high frequency of post-vitellogenic and atretic oocytes, but low frequencies of oogonia and hemocytes.

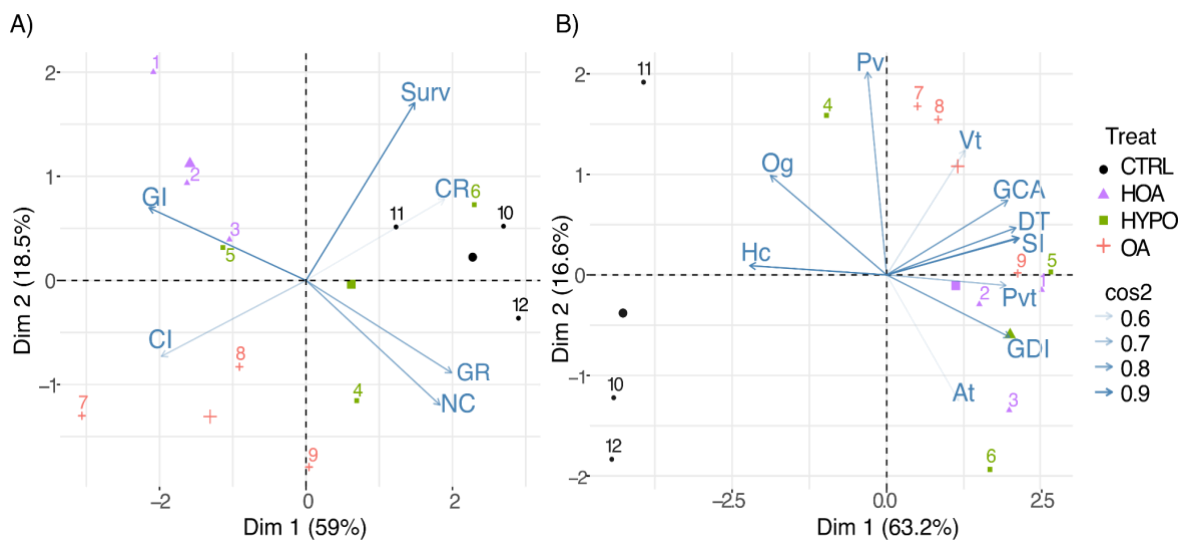


FIG. 4.4. PCA on the physiological responses (A) and gonad histology data (B) of *A. purpuratus* at the end of the experiment (Day 51). Biplots represent the first two principal components with 80.9 and 77.5% of the total variance explained for A and B, respectively. Surv: Survival, CR: Standardized clearance rate, GR: Growth rate, CI: Condition index, NC: net calcification rate, GI: Gonadosomatic index, GCA: Gonad Coverage Area, GDI: Gamete Development Index, Og: oogonia; Pv: previtellogenic oocytes, Vt: vitellogenic oocytes, Pvt: post-vitellogenic oocytes, At: atretic oocytes, Hc: hemocytes. Treatments (Treat) are color-coded, and their transparency reflects their representation quality (cos2).

4.4 Discussion

4.4.1. Effects of hypoxia and low pH on scallop survival and growth

This study suggests that medium-term (51 days) hypoxia and low pH have negative effects on the overall metabolic performance of *A. purpuratus*. The survival rate, over 90% in the stress treatments after 14 days of experimentation, fell to 46-70% after 51 days (Fig. 4.1). During this period, the negative effects of low pH were evident in several physiological measurements, in particular growth rates (Fig. 4.3). According to Thomas et al. (2019), two non-exclusive hypotheses can explain the decrease in growth when organisms are exposed to environmental stressors such as hypoxia: a decrease in energy uptake and/or an increase in energy expenditure. The decreases in clearance and calcification rates in the present study are in agreement with the first hypothesis and are consistent with another study on *A. purpuratus* juveniles which showed negative effects of hypoxia and acidification on growth, respiration and calcification rates after 11 days (19.61 mm mean shell height, water temperature of 18°C, 45% oxygen saturation and pH 7.6; Ramajo et al., 2019). Similarly, we can hypothesize an increase in energy expenditure in the event of hypoxia and low pH, linked to the maintenance of homeostasis. During the second month of conditioning, survival of the specimens exposed to the stress treatments decreased, but there was no further effect on growth rate and condition index (Table 4.3). Moreover, after 51 days, specimens from all treatments had grown well, from 12.7 mm to ca. 28.2 mm (mean shell growth rate: 304 $\mu\text{m d}^{-1}$), which is close to the values found on Peruvian scallop grounds (Aguirre-Velarde et al., 2015; Mendo et al., 2016). This apparently good growth could be linked to a certain ability of the survivors to live in such harsh conditions. The consequences of combined hypoxic and acidic conditions are usually more dramatic than those observed for single stressors (Sui et al., 2016). However, HOA treatment (combined hypoxia and OA) showed higher survival rate than single-stress treatments, suggesting potential antagonistic interaction. Such pattern has been previously recorded for other species inhabiting upwelling systems (Boch et al., 2017; Bednarsek et al., 2018; Ramajo et al., 2019). For example, warmer waters associated with ENSO events in central California acted antagonistically to lower pH, reducing the negative influence of pH on fertilization success in red abalone *Haliotis rufescens* (Boch et al., 2017).

Clearance rate decreased in all stress treatments from the start of the experiment. This result may indicate that, under these challenging conditions, individuals enter a metabolic depression that allows them to save energy for vital processes such as homeostasis (van der Veer et al., 2003). Wang and Widdows (1993) suggested that costly and non-essential physiological processes, such as digestion, absorption and growth were interrupted in *Abra tenuis* when the oxygen partial pressure was below 10 kPa (about 10% DO saturation). The oxygen saturation used in this experiment was close to the P_{cO_2} of *A. purpuratus* (Aguirre-Velarde et al., 2016) below which a decrease in oxygen consumption is observed. This decrease might be due to the prioritization of some physiological processes over others (Thomas et al., 2019). Other studies showing decreases in feeding rate and assimilation efficiency have suggested possible changes in

the cellular processes responsible for energy mobilization (e.g., Jager et al., 2006). OA has negative effects on *A. purpuratus*: for example, a pH of ca. 7.7 can lead to atrophy of the digestive gland (Lardies et al., 2017). This pattern is consistent with observations in other bivalves showing that clearance and ingestion rates are impaired by OA, probably due to deficiencies in the digestive and filtering systems (Fernández-Reiriz et al., 2012; Navarro et al., 2013; Vargas et al., 2013; Lardies et al., 2017). Reduced feeding under OA conditions has also been shown to result in lower survival, growth and calcification rates (Dupont and Thorndyke, 2009). Interestingly, lower clearance rates in the present study did not seem to have a negative effect on scallop growth in height and weight. This could result from an adaptive advantage of the survivors in favor of greater food assimilation, or from a change in diurnal feeding behavior, which could not be observed during the experiment. This result is in agreement with a previous study, which showed a transient effect of hypoxia on filtration activity, leading to a limited effect on weight growth (Aguirre-Velarde et al., 2018).

In this study, the most visible effect of stress factors on scallop metabolism was a decrease in calcification rates, in both hypoxia and acidification treatments. Decreased calcification may be due to reduced food intake and/or undersaturation of Ω calcite and Ω aragonite in seawater (Table 4.1). Considering the energy cost of calcification and organic matrix production, calcification is seen as an inexpensive and relatively simple process of carbonate ion accumulation, transport and precipitation (Palmer, 1992). However, ocean acidification forces organisms to use additional energy to maintain internal pH homeostasis/acid-base regulation, which severely impairs or alters biomineralization processes and the energy budget (Pörtner, 2008; Pan et al., 2015). Under OA stress, some marine invertebrates can increase organic carbon uptake to compensate for higher energy costs, but short-term hypoxia can weaken their ability to withstand low pH (Ravaglioli et al., 2019). Mukherjee et al. (2013) hypothesized that marine invertebrate larvae could compensate for the negative effects of OA under mild hypoxia. In *A. purpuratus*, experimental data have shown that the calcification response to OA can depend on the acidification threshold used. For example, Ramajo et al. (2016, 2019) found that calcification rate increased at pH 7.6, whereas Córdova-Rodríguez et al. (2022) and this study showed a decrease in calcification rate at pH 7.4. Waldbusser et al. (2016) suggested that in future OA scenarios, energy demand would increase to cope with the higher cost of calcification processes. Carbonate ion depletion would result in thinner, more fragile shells, making them more vulnerable to predators and environmental stressors (Feely et al., 2004). In this study, the weakness or rupture of shell hinge ligaments observed in addition to changes in shell calcification rate would affect valve mobility and reduce the ability of scallops to escape predators.

4.4.1. Effect of hypoxia and low pH on scallop maturation and reproduction

Histological data showed that, after 51 days, stressed scallops were mature, while scallops in the control treatment were still maturing (Table 4.2). Mature scallops showed good reproductive potential with high oocyte quality (Table 4.4 and Fig. 4.3). However, some differences were found between treatments: (1) scallops exposed to the multiple-stress treatment (HOA) showed high

reproductive potential when considering the abundance of different types of reproductive cells, little atresia, high GCA and GDI, good oocyte quality, and low frequency of oogonia and hemocytes. (2) Scallops exposed to single-stress treatments (HYPO, OA) had also high reproductive potential and good oocyte quality, but with higher frequency of oogonias. (3) Scallops in the control condition (CTRL) exhibited a very different reproductive pattern, that is gonad immaturity (high frequency of oogonias and hemocytes). High frequency of oogonias in some treatments may indicate the beginning of a new oocyte genesis: in Peruvian bays, *A. purpuratus* is a continuous spawner with almost monthly reproductive cycles (Wolff, 1988; Aguirre-Velarde et al., 2019). The presence of hemocytes may signify healthy reproductive processes, as the former contribute to the elimination of residual oocytes in the gonad (Beninger, 2017). In marine bivalves, high frequency of atretic oocytes and intense hemocyte infiltration do occur during periods of intense reproductive activity (Beninger, 2017; García-Corona et al., 2018) or gonad ripening under detrimental environmental conditions (Rodríguez-Jaramillo et al., 2022). In this study, the higher frequency of atretic oocytes in the stress treatments, compared with the control condition, may be related to advanced maturation. Oocyte atresia, i.e. the reabsorption of vitellogenic oocytes in the gonads (Hunter and Macewicz, 1985), may also be a strategy in pre-spawning organisms, allowing bivalves to halt the reproductive process when unfavorable environmental conditions could compromise larval development (Beninger, 2017; Rodríguez-Jaramillo et al., 2022). The present study showed stimulation of gonad maturation with high reproductive potential in stress treatments, but low amounts of oogonias, particularly in the HOA treatment. The latter may indicate a subsequent disruption in the reproductive cycle of these scallops.

Mendo et al. (1987) found that *A. purpuratus* reaches maturity at a shell height of ca. 25 mm, which is close to the individual size at the end of this study. Although gonadal maturation was not continuously monitored in this study, there are indications that stress factors stimulated early maturation. To explain this trend, we formulate four hypotheses, which may or may not be interrelated:

- (1) The stress conditions may reduce maximum body size and subsequently size at maturity. Cardoso et al. (2006) suggested that first reproduction in bivalve species is linked to maximum body size: on average, female bivalves would become mature at about 15% of their maximum body size. The latter hypothesis could not be verified in the present study due to the relatively short duration of the experiment;
- (2) Environmental stress conditions would speed up the individual aging process (Andreyeva et al., 2021), through the production of oxidative stress that induces progressive and irreversible oxidative damage in the cell. As oxidative stress regulation or tolerance is closely related with longevity (Blier et al., 2017), stressed scallops may age and mature earlier despite their size, as observed in *A. ventricosus* (García-Corona et al., 2020; Mazón-Suastegui et al., 2021);
- (3) Individuals in stress conditions would have matured earlier than scallops of the control treatment due to adaptive responses called hormesis. Hormesis refers to adaptive responses of biological systems to mitigate environmental or self-imposed challenges through which the system improves its functionality and/or tolerance to more severe challenges

(Calabrese and Mattson, 2017). Mito-hormesis (mitochondrial hormesis) has been documented as a mitochondrial response to exogenously induced punctual stress events that triggers a compensatory retrograde response by increasing mitochondrial activity and initiating antioxidant systems (Ristow and Zarse, 2010). Mitohormesis could be particularly important in invertebrates, which are expected to react rapidly to environmental cues (Buttemer et al., 2010). However, this process may no longer apply under prolonged adverse conditions;

- (4) Another explanation could be that early maturation is an emergency reproductive strategy under unfavorable environmental conditions, which can help mitigate the effects of stressors at the population scale. In scallops, starting maturation earlier (and therefore spawning) would be a subsistence strategy to ensure offspring (García-Corona et al., 2020; Mazón-Suastegui et al., 2021). However, reproductive potential is lower in this case due to the smaller size of the gonads. Furthermore, prioritizing reproduction over defense and escape capacities is dangerous (Brokordt et al., 2013) although very common for fast-growing species such as *A. purpuratus*. When individuals reach a larger volume, the demand for energy to maintain homeostasis is higher. Size reduction and early reproduction could therefore be a strategy for coping with life under unfavorable environmental conditions (Pecquerie et al., 2009; García-Corona et al., 2020; Pauly, 2021).

Maintaining a small size and reproducing at a reduced size can have a significant impact (Haag, 2013) on wild and cultivated populations of *A. purpuratus* along the Humboldt Current System. The reproductive potential and spawning cycles of scallops would be affected (Aguirre-Velarde et al., 2019) and recruitment would be reduced (Kluger et al., 2019). A smaller size could also affect demand, market value and producers' income. Knowing the negative effects of extreme and prolonged environmental stressors on the species, growers must be able to practice adaptive cultivation. For example, they can apply technologies that oxygenate hypoxic bottoms or move scallops from the bottom to more superficial layers (suspended culture; Cueto-Vega et al., 2021).

4.5 Conclusions

This experimental study was set up under conditions of environmental stress that reproduced the persistent extreme conditions (severe and chronic hypoxia) already present in Peruvian bays. Despite certain limitations concerning exposure duration and physiological measurements, the results provide new ecophysiological insights into how the scallop species *Argopecten purpuratus* responds to the prolonged combination of seawater hypoxia and acidification. This experiment suggests different metabolic trade-offs where maturation takes priority over the formation of body structures, such as somatic tissue and shell. Survivors showed relatively good physiological performances in coping with prolonged hypoxia and low pH, consistent with previous works suggesting that *A. purpuratus* is acclimatized to cold, acidic and hypoxic conditions of short duration. Nevertheless, a smaller size may significantly affect wild and cultivated scallops at the population level. Further studies are needed to better understand the response mechanisms and bioenergetic processes involved in maintaining

homeostasis. We also need to develop applications of the scallop DEB model that better explain the priority given to gonad maturation over growth under unfavorable environmental conditions (linked, for example, to intensified upwelling or ENSO events).

4.6 Acknowledgments

We would like to thank IMARPE, and in particular John Dionisio and Mariandrea Rosado of the Aquatic Ecophysiology Laboratory, Gheraldine Inga of the Phytoplankton Laboratory, as well as Wilson Carhuapoma and Michel Graco of the Chemical Oceanography Laboratory. Their invaluable assistance in providing laboratory facilities, cultivating cultures, and conducting chemical analyses of seawater was greatly appreciated. At LEMAR, we would also like to thank Nelly Le Goïc for providing the laboratory facilities required for histological analyses. This work is part of a PhD thesis supported by the French National Research Institute for Sustainable Development (IRD) within the frameworks of the LMI DISCOH, the JEAI DYSRUP and the GDRi DEXICOTROP. The CNRS-PICS BISCOT also supported it. The lead author was supported by a grant from the Franco-Peruvian Doctoral School in Life Sciences.

Chapter 5

Protein regulation response under combined hypoxia and ocean acidification in the Peruvian scallop *Argopecten purpuratus* (Lamarck, 1819)

Comparative Biochemistry and Physiology - Part D : Genomics and Proteomics
(in prep.)

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Abstract

Argopecten purpuratus is an important economic resource in Peru. In recent years, mass mortalities of bivalves in Peruvian coastal waters have threatened this resource. These mortalities are caused by hypoxic events, which occur when oxygen levels in seawater drop below 0.5 mg/L. Other related environmental processes, such as acidification and sulfur plumes, also contribute to the threat. Hypoxia and ocean acidification (OA) are strongly correlated and can have a negative impact on scallop performance. To understand the molecular mechanisms underlying the response of *A. purpuratus* to these stressors, a 2x2 factorial design experiment was conducted. After 51 days, a comparative proteomic analysis of the mantle tissue was performed. The analysis revealed significant accumulation of 27 proteins between hypoxic and acidified samples. Animals exposed to a single stressor (hypoxia or acidification) showed a different response compared to those exposed to both stressors. Animals exposed to a single stressor accumulated a large amount of proteins, while those exposed to both stressors showed a deregulation strategy prioritizing proteins related to energy production and homeostasis. Four stress markers (Pah, Aldoa, Smp 194770, and Odh) are proposed for *A. purpuratus* under hypoxia and OA. This is the first proteomic analysis to provide insights into the link between long-term acclimation and protein regulation under combined hypoxia and OA in *A. purpuratus*.

Keywords : Hypoxia, reduced pH, adaptation, homeostasis, protein synthesis.

5.1 Introduction

The Peruvian scallop, *Argopecten purpuratus*, is an important filter-feeding resource in Peru and Chile. Scallop farming in Peru showed significant advantages in the early 2000s compared to Chile because of lower production costs and the high availability of seeds on natural beds (Kluger et al., 2019). Nevertheless, in the last decade, huge scallop mass mortalities recorded in Sechura (5°40' S, 80°54' W; Piura) and Paracas (13°50' S, 76°16' W; Ica), two of the main scallop culture areas in Peru, negatively impacted the production (Aguirre-Velarde et al., 2019; Cueto-Vega et al., 2021, chapter 2). For example, producers suffered economic losses over two years in 2017-2018 due to scallop mass mortalities in Sechura Bay in the year 2017 and the lack of seed supply and difficulties in getting loans to start a new grow-out cycle in the year 2018 (Kluger et al., 2019). During the last eight years, mass mortality events have been frequent and have been linked to environmental stressors such as temperature, hypoxia and bottom oxygen-depleted processes (eutrophication, red tides and hydrogen sulfide-enriched waters; Sanchez et al., 2015; Morales, 2017; Aguirre-Velarde et al., 2019; Kluger et al., 2019; Cueto-Vega et al., 2021, Chapter 2).

The Peruvian Humboldt Current System (HCS) is associated with a strong oxygen minimum zone (OMZ; permanent hypoxic areas in the deep ocean; Alheit & Niquen, 2004). Depending on years, sporadically advected OMZ water and/or high productivity associated with the coastal upwelling can lead to hypoxia and high pCO₂ in shallow coastal waters (Chavez et al., 2008; Graco et al., 2017). Likewise, in the HCS, stronger upwellings may generate a minor water column stratification with low temperatures, pH, and oxygen conditions (Ramajo et al., 2020). This natural forcing, along with the impacts of anthropogenic perturbations, is magnified by climate change and may have led to more hypoxic and acidic coastal seawater in recent years (Graco et al., 2017; Altieri & Diaz, 2019). Therefore, marine organisms face unprecedented threats from multiple stressors associated with climate change, particularly deoxygenation and acidification of the ocean (Vaquer-Sunyer & Duarte, 2008; Hofmann et al., 2010; Gunderson et al., 2016).

Experimental studies have demonstrated that *A. purpuratus* individuals from the northern and central HCS exhibit a relatively high tolerance to specific stressors, such as hypoxia (Aguirre-Velarde et al., 2016, 2019; Cueto-Vega et al., 2021) and ocean acidification (Ramajo et al., 2016; Lagos et al., 2016; Lardies et al., 2017; Córdova-Rodríguez et al., 2022). However, it is worth noting that extended periods of intense upwelling events can lead to significant scallop mortalities in the northern Chilean (Ramajo et al., 2020) and Peruvian (Aguirre-Velarde et al., 2019) cultured populations. Severe hypoxia has also been linked to detrimental effects on the growth, reproduction, and survival of Peruvian scallops (Aguirre-Velarde et al., 2019). Laboratory experiments have documented reduced clearance rates (Aguirre-Velarde et al., 2018) and respiration rates in Peruvian scallops under hypoxic conditions (Aguirre-Velarde et al., 2018, Chapter 4). Similarly, adverse impacts on growth and net calcification due to low pH were observed after a 30-day exposure (Córdova-Rodríguez et al., 2022). A recent unpublished study suggests that when exposed to prolonged hypoxic and acidic conditions, scallops reduce their feeding activity, prioritizing energy allocation towards gonad maturation (Cueto-Vega et al. *unpublished*).

Modern scientific techniques, such as -omics approaches, provide the opportunity for a comprehensive understanding of how organisms respond to environmental stressors and the underlying mechanisms involved. Specifically, the proteome, a dynamic and intricate network of proteins, can swiftly react to environmental influences, often independently of the genome (Tomanek, 2014). The changes observed in the proteins within the proteomic profile enable us to comprehend the signaling events that transform environmental stress into a biochemical response at the cellular level (Tomanek, 2014). For example, in bivalves exposed exclusively to hypoxia, an increase in antioxidant defenses in response to oxidative stress, as well as heightened activity of mitochondrial proteases responsible for the breakdown of damaged mitochondrial proteins have been reported (Andreyeva et al., 2021; Sokolov et al., 2019). Another study analyzing the response to hypoxia in the great scallop (*Pecten maximus*) suggests the need for energy conservation and a role for apoptosis at temperatures of 18 and 25°C (Artigaud et al., 2015). Similarly, exposure to reduced pH or ocean acidification resulted in increased energy demands, leading to disruptions in carbohydrate metabolism, cell growth, cytoskeletal dysfunction, protein synthesis, and extracellular matrix components in oysters like *Crassostrea gigas* (Wei et al., 2015), *Saccostrea glomerata* (Thompson et al., 2016), and the geoduck *Panopea generosa* (Timmins-Schiffman et al., 2020). There are only few studies that analyzed the effect of the combined hypoxia and acidification in bivalves at cellular level, for example, have been reported an increased production of antioxidant enzymes in response to oxidative stress in the mussel *Mytilus coruscus* (Sui et al., 2016; Khan et al., 2021). Nevertheless, this response is apparently time-dependent because after 30 days of exposure, the activities of antioxidant enzymes decrease in *M. coruscus* due to a reduced capacity to cope against the production of reactive oxygen species (ROS) (Khan et al., 2021).

While the impact of environmental stressors on *A. purpuratus* at cellular level remains unexplored, a prior study conducted at the transcriptomic analysis to explore the response of post-larvae exposed to some contaminants such as copper (Details in Zapata et al., 2009). The objective of the current work was to determine, using a proteomic approach, whether prolonged exposure (51 days) to combined stressors of hypoxia and ocean acidification exerts more severe negative effects on the mantle of *A. purpuratus* than exposure to individual ones. One of the primary advantages of a proteomic approach, beyond the identification of stress markers, is its ability to decipher the functional responses related to environmental stress, thus providing insights into scallop health and its aquaculture in the context of high environmental variability.

5.2 Material and Methods

5.2.1 Sampling and acclimation procedure

Juvenile scallops (n = 1500, age : 3 months) were provided from the hatchery AgroMar del Pacífico (Piura, Peru) and transferred in a cool box to the Aquatic Ecophysiology Laboratory of IMARPE (Callao, Peru). Once in the laboratory, scallops were distributed into eight 64-L aquaria continuously provided with filtered (10 µm), thermoregulated (approx. 16°C) and oxygen-saturated (aprox. 100%) seawater with a flow of 7.3 L h⁻¹. After an acclimation period of one week (to reduce stress generated by transport and manipulation), scallops of similar

size (12.7 ± 1.6 mm SD shell height) were randomly distributed into 12 independent rectangular 44-L aquaria to start the experiment.

5.2.2 Experimental design and Methods

Scallops were exposed to challenging and normal conditions of oxygen (hypoxia and normoxia) and pH (acidic and normal pH) during 51 days. The oxygen challenging condition consisted of exposing scallops to 20% oxygen saturation, which is the level at which scallops enter in their oxy-conformity phase (Aguirre-Velarde et al., 2016 ; Cueto-Vega et al., 2021). The acidic condition consisted in an exposure to a pH of 7.4 already used in a precedent study that reported negative effects of low pH in the Peruvian scallop (Córdova-Rodríguez et al., 2022). The control conditions values (high oxygen saturation at 100% and high pH at 7.8) are consistent with values found in Peruvian coastal bays (Carhuapoma, pers. comm.), especially during the Austral summer.

The exposition consisted in four treatments : control under normoxia and normal pH (CTRL), hypoxia only (HYPO), OA only (OA) and combined hypoxia and OA (HOA). Once acclimated, juvenile scallops were exposed to the experimental treatments by triplicate. In total, 1080 specimens were randomly divided in 12 groups of 90 individuals and transferred into 44-L aquaria.

Low oxygen saturation in hypoxic conditions (HOA and HYPO) within the experimental tank was controlled by Nitrogen injection into the seawater. An oxygen probe (FDO 925-3 dissolved oxygen probe, WTW, USA) continuously measured the oxygen concentration and temperature within the experimental aquariums. The probe was interfaced with a computer that controlled the injection of nitrogen through a solenoid valve, which allows control of the oxygen concentration within each aquarium. Normoxia treatments were aerated by bubbling air to reach ca. 100% during the experiment. Low pH treatments were achieved by bubbling seawater with CO₂ until the pH of 7.4 is reached in the seawater. Controlled CO₂ addition in reduced pH treatments was achieved using a pH controller (Tunze 7070/2, Germany) attached to a CO₂ bottle that released a suitable amount of the gas into a 400-L header tank. From this header tank, the filtered seawater (10-µmplus UV filter) flowed continuously (5.3 L h) to the six aquaria with low pH conditions. Normal pH seawater (i.e ca. 7.8), was filtered and provided directly at the same flow rate to the six normal pH aquaria.

In all the aquaria pumps allowed homogenization and temperature was maintained constant at 16°C using a chiller. In order to avoid gas exchanges, all the aquaria were covered with polystyrene sheets. Oxygen saturation, temperature and pH measurements were measured every day in each aquarium using a multi-parameter (WTW multi 3430) connected with an optical oxygen sensor (FDO 925 dissolved optical oxygen probe) and a pH electrode (Sentix® 940), respectively. The pH electrode was calibrated daily on NBS scale (pH 4 and 7). Scallops in each aquarium were fed continuously using peristaltic pumps with an algae mixture (*Isochrysis galbana* and *Chaetoceros calcitrans* 1:1) to maintain a concentration ca. 9000 cells mL. All the aquaria were cleaned daily with a siphon for removing feces, in addition, dead scallops were removed from the tanks. After 51 days, five scallops (randomly selected) from each treatment (20 in total) were dissected and weighted. Immediately, the mantle tissues were frozen (-70°C), lyophilized and kept frozen until proteomic analysis.

5.2.3 Total protein extraction

Total proteins were extracted from the lyophilized mantle tissues (approximately 100 mg per sample) by adding 500 μ L extraction buffer (2M Thiourea, 7M Urea, 4% CHAPS, 50 mM TrisHCl pH 8.5 with 1% protease inhibitor mix ; GE Healthcare) and cold crushing using a Fastprep™ 5G Instrument (3 cycles of 40s with 60 s rest). Then, the samples were centrifuged at 15 000 x g for 20 min at 4 °C. The supernatant was transferred to a clean tube and proteins were quantified (Bradford, 1976).

5.2.4 LC-MS/MS analysis

Five μ g of each protein extract were prepared using a modified Gel-aided Sample Preparation protocol (Fischer and Kessler, 2015). Samples were digested with trypsin/Lys-C overnight at 37°C. For nano-LC fragmentation, protein or peptide samples were first desalted and concentrated onto a μ C18 Omix (Agilent) before analysis. The chromatography step was performed on a NanoElute (Bruker Daltonics) ultra-high-pressure nano flow chromatography system. Approximately 200 ng of each peptide sample were concentrated onto a C18 pepmap 100 (5mm x 300 μ m i.d.) precolumn (Thermo Scientific) and separated at 50°C onto a reversed phase Reprosil column (25cm x 75 μ m i.d.) packed with 1.6 μ m C18 coated porous silica beads (Ionoptix). Mobile phases consisted of 0.1% formic acid, 99.9% water (v/v) (A) and 0.1% formic acid in 99.9% ACN (v/v) (B). The nanoflow rate was set at 400 nl/min, and the gradient profile was as follows : from 2 to 15% B within 60 min, followed by an increase to 25% B within 30 min and further to 37% within 10 min, followed by a washing step at 95% B and reequilibration. MS experiments were carried out on an TIMS-TOF pro mass spectrometer (Bruker Daltonics) with a modified nano electrospray ion source (CaptiveSpray, Bruker Daltonics). The system was calibrated each week and mass precision was better than 1 ppm. A 1600 spray voltage with a capillary temperature of 180°C was typically employed for ionizing. MS spectra were acquired in the positive mode in the mass range from 100 to 1700 m/z. In the experiments described here, the mass spectrometer was operated in PASEF mode with exclusion of single charged peptides. A number of 10 PASEF MS/MS scans were performed during 1.25 seconds from charge range 2-5. Before post-process, the samples are analyzed using Preview software (ProteinMetrics) in order to estimate the quality of the tryptic digestion and predict the post-translational modifications present. The result, below, is used for the "bank research / identification" part. The fragmentation pattern was used to determine the sequence of the peptide. Database searching was performed using the Peaks X+ software (Ma et al., 2003).

A *Mizuhopecten yessoensis* database was used. The variable modifications allowed were as follows : K-acetylation, methionine oxidation, Deamidation (NQ), Methylation and Dimethylation (KR) and Carbamylation. In addition, C-Propionoamide was set as a fixed modification. "Trypsin " was selected as Specific. Mass accuracy was set to 30 ppm and 0.05 Da for MS and MS/MS mode, respectively. Data were filtering according to a FDR of 1% and the elimination of protein redundancy on the basis of proteins being evidenced by the same set or a subset of peptides. To quantify the relative levels of protein abundance between different groups, samples were analyzed using the label-free quantification feature of PEAKS X+ software. Feature detection was

separately performed on each sample by the expectation-maximization based algorithm. The features of the same peptide from all replicates of each sample were aligned through the retention time alignment algorithms. Mass error tolerance was set at 30 ppm, Ion Mobility Tolerance (1/k0) at 0.07 and retention time tolerance at 10 min. Normalization factors of the samples were obtained by the total ion current (TIC) of each sample. Quantification of the protein abundance level was calculated using the sum area of the top three unique peptides.

5.2.5 Data analysis

Label-free quantitative data were imported into Perseus in which statistical analyses were performed ((Tyanova et al., 2016). Protein intensity was log₂-transformed and only proteins identified in every sample of at least one of the conditions tested were kept for further analysis. Data were then compared using a multiple t-test between the conditions (HOA, HYPO, OA and CTRL, significance level : $p = 0.05$; FDR = 0.05). To observe the changes in the protein profiles by treatment, a heat map was constructed by using standardized log₂-intensities.

Then, to annotate the proteins, we used proteins with significant differences (tested above) to perform a BLASTp search (NCBI ; www.ncbi.nlm.nih.gov), with an e-value cut-off 10^{-3} and region of sequence similarity covered greater than 98%. Gene Ontology (GO) for candidate genes was assessed using UniProt (<http://www.uniprot.org/>) and QuickGO (<https://www.ebi.ac.uk/QuickGO>). A network analysis was performed, to explore the relationships between the proteins, with the proteins mentioned above through STRING v. 11.5 (<http://string-db.org>) using the *Danio rerio* database.

Then, to explore any relation between log-protein intensities, their treatments and their functional roles, we performed principal component analysis (PCA). For the PCA, the variables (previously scaled to be zero-centered) were assessed by using the “factoextra” R package (Kassambara and Mundt, 2020) in R software (version 4.1.2 ; R Core Team, 2021).

5.3 Results

Mortality after 51 days of experiment was in average of 53.8 ($\pm 9.9\%$ SD), 37.8 ($\pm 23.3\%$ SD), 29.7 ($\pm 1.5\%$ SD) and 12.0 ($\pm 7.6\%$ SD) in OA, HYPO, HOA and CTRL treatments, respectively. After the experiment, the proteomic analysis results of the survivors' mantle are presented below.

5.3.1 Differential proteomics profiles between treatments

In total 1871 proteins were identified, from these, 1539 were present in all the replicates of at least one of the four treatments analyzed (HOA, HYPO, OA and CTRL). In total 27 proteins were found significantly (multiple t-test, corrected with FDR < 0.05) accumulated or deregulated in at least one treatment. Proteins were functionally annotated by examining their associated Clusters of Orthologous Group (COG) categories, biological process GO terms, and literature review (Details in Table 5.1). Proteins are discussed below according to their potential roles in different aspects of hypoxia and acidic stress as : redox homeostasis (RH), protein metabolism (PM), immune response (IR), and energy production (EP). General profiles on proteins show accumulation/deregulation patterns,

especially between the HOA with HYPO and HYPO (Fig. 5.1). Thereby, the profiles for each treatment show a significant accumulation of proteins in the OA and HYPO treatments (13/27 and 12/27 proteins, respectively). Whereas 13/27 proteins were significantly deregulated ($p < 0.05$, multiple t-test) in HOA (Table 5.1).

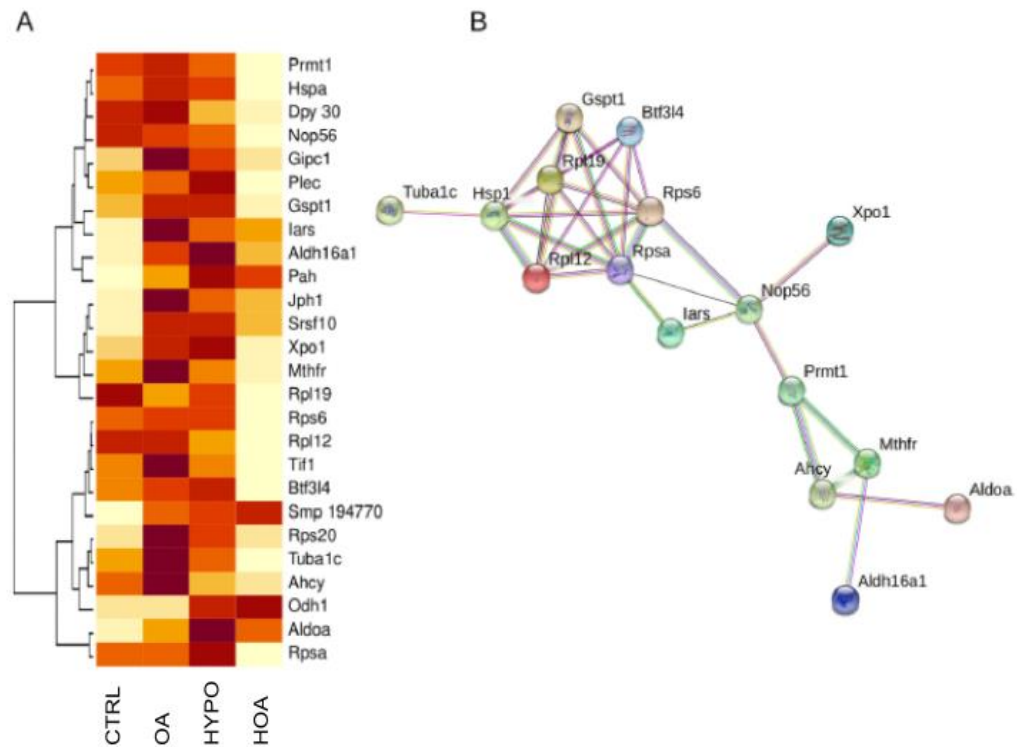


FIG. 5.1: Overview of differentially expressed (DE) proteins associated with 51 days of challenging conditions under hypoxia and ocean acidification (OA) in *A. purpuratus* tissues. It includes two main components: A) A heatmap displaying the \log_2 -protein intensities of 27 DE proteins, and B) Network analysis of the identified proteins, constructed using STRING v. 11.5 based on known protein interactions in the model organism *Danio rerio*. The line numbers and colors, with color codes explained at <http://string-db.org>, depict the strength and nature of interaction evidence for various proteins. The mentioned proteins, such as Ahcy (adenosylhomocysteinase B), Aldh16a1 (aldehyde dehydrogenase family 16 member A1), Aldoa (fructose-bisphosphate aldolase), Btf3l4 (transcription factor BTF3 homolog 4), Dpy_30 (protein dpy-30 homolog isoform 4), Gspt1 (eukaryotic peptide chain release factor subunit 3a), Gipc1 (PDZ domain-containing protein), Hspa14 (heat shock 70 kDa protein 4), Iars (isoleucine-tRNA ligase, cytoplasmic), Jph1 (junctophilin-1), Plec (plectin isoform X4), Mthfr (methylenetetrahydrofolate reductase), Nop56 (nucleolar protein 56), Odh1 (octopine dehydrogenase), Pah (phenylalanine-4-hydroxylase), Prmt1 (protein arginine N-methyltransferase 1), Rpl12 (60S ribosomal protein L12), Rpl19 (60S ribosomal protein L19), Rps20 (40S ribosomal protein S20), Rps6 (40S ribosomal protein S6), Rpsa (40S ribosomal protein SA), Smp_194770 (taurocyamine kinase), Srsf10 (serine/arginine-rich splicing factor 10), Tif1 (ATP-dependent RNA helicase eIF4A), Tuba1c (tubulin alpha-1 chain), Xpo1 (exportin-1).

There were two proteins identified as accumulated in the 3 stress treatments (Table 5.1) : taurocyamine kinase (Smp 94770 ; fold-change of at least 1.5) y phenylalanine-4-hydroxylase (Pah ; fold-change of at least 3.3), related with

energy production and redox homeostasis. The single stress treatments (HYPO and OA) show a greater concentration of exportin-1 (Xpo1), 40S ribosomal protein S20 (Rps20), isoleucine-tRNA ligase (Iars), serine/arginine-rich splicing factor 10 (Srsf10), Eukaryotic peptide chain release factor subunit 3a (Gspt1), aldehyde dehydrogenase family 16 member A1 (Aldh16a1), junctophilin-1 (Jph1), PDZ domain-containing protein (Gipc1) and plectin isoform X4 (Plec). Among proteins with oxido-reductase activity, Aldh16a1 was the most abundant with a fold-change of 16.7 and 8.1 in the HYPO and OA treatment, followed by PAH with a fold-change of 8.8 and 5.7 in the HYPO and HOA treatment.

The grouping of accumulated proteins linked to energy production have an evident relation to the hypoxia and combined hypoxia treatments (Fig. 5.3). The latter grouping is clearly separated to the others, showing an inverse relation with the redox homeostasis grouping (Fig. 5.3). Thereby, Octopine dehydrogenase (Odh) and fructose-bisphosphate aldolase (Aldoa), linked to energy production, were more abundant in the hypoxia treatments with a fold-change of at least 1.5 (Table 5.1). In HYPO and HOA, the protein dpy-30 homolog isoform 4 (Dpy30) linked to RH were significantly ($p < 0.05$, t-multiple test) deregulated (Table 5.1). The homeostasis-related protein methylenetetrahydrofolate reductase (Mthfr) (fold-change of 2.9) was remarkably accumulated in the OA treatment (fold-change of 3.3). Contrary, there were 13 proteins (Gipc1, Rpsa, Tuba1c, Rps6, Tif1, Rpl12, Btf3l4, Ahcy, Hspa14, Rpl19, Dpy30, Nop56 and Prmt1) were significantly deregulated ($p < 0.05$, t-multiple test) in the HOA treatment with a fold-change between 0.3 and 0.8.

5.3.2 Network Analysis

A network analysis using STRING was performed on 27 proteins for which we obtained significant differences. Network analysis highlighted relationships between 15 of them (Fig. 5.1B). The largest network of differentially accumulated proteins in this study connects proteins involved in protein metabolism (60S ribosomal protein L12, Rpl12 ; 60S ribosomal protein L19, Rpl19 ; 40S ribosomal protein S20, Rps20 ; 40S ribosomal protein S6, Rps6 ; 40S ribosomal protein SA, Rpsa ; nucleolar protein 56, Nop56). Most of them interact with the transcription factor Btf3 homolog 4 (4 interactants) and the eukaryotic peptide chain release factor subunit 3a (4 interactants) related to apoptotic processes. Followed by Hspa14 (4 interactants), Prmt1 (4 interactants), Mthfr (4 interactants), Ahcy (4 interactants) and Aldh16a1 (1 interactant) related with redox homeostasis and immune response. In the network, the energy production-related protein Aldoa has 1 interaction with Mthfr. Despite the relationship of interactants in the network, profiles of DE proteins in each stress treatment (OA, HYPO and HOA) show different expression patterns (Fig. 5.1). Additionally, two DE proteins (from 27) present in all stress treatments (PAH and Smp 94770) did not belong to the network.

TAB. 5.1: List of proteins significantly different from the control (CTRL), identified in the mantle tissue of *Argopecten purpuratus* after 51 days of experiment under hypoxia and reduced pH conditions.

CTRL	Protein intensity (Log ₂)			Protein ID (<i>Mizuhopecten yessoensis</i>)	Protein name	COG	GO term of Biological process	Functional role discussed
	OA	HYPO	HOA					
17.91 (ac)	18.55 (a)	17.42 (c)	17.11 (b)	XP 021357992.1	Ahcy	F	GO:0004013	RH: Antioxidant response
13.12 (a)	16.14 (b)	17.18 (b)	14.44 (a)	XP 021359418.1	Aldh16a1	F	GO:0016491	RH
20.01 (a)	20.42 (ab)	20.96 (c)	20.57 (bc)	XP 021345402.1	Aldoa	F	GO:0003824	EP
18.38 (a)	18.75 (a)	18.85 (a)	17.60 (b)	XP 021346552.1	Btf3l4	C	GO:0005576	IR: Apoptotic processes
15.61 (a)	15.71 (ac)	14.35 (cb)	13.75 (b)	XP 021367614.1	Dpy 30	C	GO:0048188	RH
14.48 (a)	15.89 (b)	15.29 (b)	14.23 (a)	XP 021342310.1	Gipc1	F	GO:0004957	IR: Immune-pathway activation
16.19 (ab)	16.86 (b)	16.85 (b)	15.86 (a)	XP 021361578.1	Gspt1	F	GO:0003924	IR: Apoptotic processes
15.03 (a)	15.72 (a)	15.30 (a)	13.41 (b)	XP 021346238.1	Hspa14	P	GO:0006457	RH: Antioxidant response
15.02 (a)	16.19 (b)	15.74 (b)	15.55 (ab)	XP 021343309.1	Iars	F	GO:0000049	PM
12.37 (a)	14.17 (b)	13.37 (ab)	12.89 (a)	XP 021343474.1	Jph1	C	GO:0030314	IR: Cytoskeleton- associated immune response
12.66 (a)	14.17 (b)	12.79 (ab)	11.72 (a)	XP 021356251.1	Mthfr	P	GO:0055114	RH
16.04 (a)	15.93 (a)	15.72 (a)	14.07 (b)	XP 021371922.1	Nop56	F	GO:0016787	PM
16.28 (a)	16.37 (a)	17.48 (b)	17.74 (b)	XP 021354844.1	Odh1	F	GO:0016491	EP
13.18 (a)	14.71 (b)	16.31 (c)	15.69 (bc)	XP 021366787.1	Pah	F	GO:0004497	RH
14.73 (ab)	15.03 (b)	15.40 (b)	14.13 (a)	XP 021373314.1	Plec	C	GO:0005856	IR: Cytoskeleton- associated immune response
15.40 (a)	15.82 (a)	15.11 (a)	13.41 (b)	XP 021345838.1	Prmt1	F	GO:0016274	IR: Immune-pathway activation
18.87 (a)	18.83 (a)	18.56 (ab)	18.21 (b)	XP 021342614.1	Rpl12	C	GO:0005840	PM
14.48 (a)	13.65 (a)	14.19 (ab)	12.68 (b)	XP 021373060.1	Rpl19	C	GO:0005840	PM
17.2 (ac)	17.9 (b)	17.7 (c)	17.2 (ac)	XP 021340759.1	Rps20	F	GO:0050324	PM
18.83 (a)	18.92 (a)	18.92 (a)	18.24 (b)	XP 021358534.1	Rps6	C	GO:0005840	PM
20.43 (a)	20.41 (a)	20.56 (a)	20.10 (b)	XP 021346605.1	Rpsa	C	GO:0005840	PM
18.35 (a)	18.94 (c)	19.02 (d)	19.13 (b)	XP 021380003.1	Smp 194770	F	GO:0005524	EP
11.92 (a)	13.93 (b)	14.02 (b)	12.80 (a)	XP 021378562.1	Srsf10	P	GO:0043484	IR: Apoptotic processes
18.74 (a)	19.23 (a)	18.72 (a)	18.11 (b)	XP 021377711.1	Tif1 or Trim24	F	GO:0006413	IR: Apoptotic processes
17.42 (ab)	17.79 (a)	17.54 (a)	17.04 (b)	XP 021370651.1	Tuba1c	F	GO:0003924	IR: Cytoskeleton- associated immune response
12.51 (a)	14.24 (b)	14.38 (b)	12.06 (a)	XP 021363249.1	Xpo1	P	GO:0006886	RH

The treatments were single hypoxia (HYPO), single acidification (OA), combined hypoxia and acidification (HOA). Proteins are classified into the following categories: IR (Immune response), EP (Energy production), PM (Protein metabolism), and RH (Redox homeostasis).

5.4 Discussion

5.4.1 Mechanisms to respond hypoxia

Hypoxia is one of the main limiting factors in the metabolism of marine organisms (Wang and Widdows, 1993). Concerning *A. purpuratus*, mortality under severe hypoxia has been recorded in the field (Aguirre-Velarde et al., 2019) and in laboratory experiments (Cueto-Vega et al., 2021, Chapter 2). In our study, negative effects of hypoxia were clearly shown, as scallops challenged with this stressor had a mortality rate of more than the double of the control group ($37.8 \pm 23.3\%$ SD, vs $12.0 \pm 7.6\%$ SD). Surviving scallops showed a relative acclimation to cope with hypoxia by accumulating several proteins (Fig. 5.1, Table 5.1). The mantle border of bivalves is an essential organ and forms inhalant and exhalant siphons, which regulate the influx and exit of seawater necessary to move food particulate material that will be filtered in the gill. It can be extended outside the animal and move actively, playing important roles in feeding, defence and reproductive processes (Ram et al., 1999). Adjustment of mantle cavity oxygen partial pressure to lower than ambient levels through controlled pumping prevents high oxygen gradients between bivalve tissues and surrounding fluid, limiting oxygen flux across the body surface (Abele et al., 2010).

The proteomic profiles in HYPO treatment showed the utilization of anaerobic metabolism by the accumulation of octopine dehydrogenase, this molecule has been reported in other bivalves exposed to hypoxia, during escape swimming or during burrow (Carroll and Wells, 1995 ; Brokordt et al., 2000 ; Strahl et al., 2011 ; van Os et al., 2012 ; Artigaud et al., 2015). Odh is an enzyme which, during anaerobic respiration, catalyzes the reductive condensation of glycolytic pyruvate and arginine into octopine, NAD^+ and water (Storey and Storey, 2004). NAD^+ is an important reductant used in glycolysis and allows for the continued production of ATP in the absence of oxygen (van Os et al., 2012). In mantle muscle, Odh is significantly correlated with a species' ability to buffer the acidic end-products of anaerobic metabolism (Seibel et al., 2000). Some bivalves rely mainly on aerobic metabolism even during severe hypoxia (Van den Thillart et al., 1992 ; Wang and Widdows, 1993). The role of Odh and lactate dehydrogenase (Ldh : an enzyme which catalyzes the conversion of lactate to pyruvate and back, as it converts NAD^+ to NADH and back within aerobic metabolism) depends on the tissue analyzed ; for example, in *Artica islandica* during anoxia and reoxygenation more Odh is accumulated in the muscle (Odh / Ldh ratio= 0.95) ; while in hemocytes is the contrary (Odh / Ldh ratio= 0.35 ; Strahl et al., 2011). Hypoxia and energy production were also closely related through accumulation of Odh, Aldoa and Smp 194770 (Fig. 5.3). Fructose-bisphosphate aldolase A (Aldoa) plays a key role in glycolysis and gluconeogenesis, which is consistent with an active aerobic metabolism. Even Though, an accumulation of Aldoa may suggest higher glycolytic rate related to anaerobic metabolism. Smp 94770, as others phosphagen kinase, is essential to ATP production process required to support the high demand animal's immune response to abiotic stress (Coyne, 2011). The accumulation of Aldoa, Smp 94770 along with the Odh1 in the hypoxia treatments may indicate that at 20% oxygen saturation, the aerobic and anaerobic pathways works at the same time in the *A. purpuratus*; which is consistent with a report in the bivalve *Abra tenuis* (Wang and Widdows, 1993).

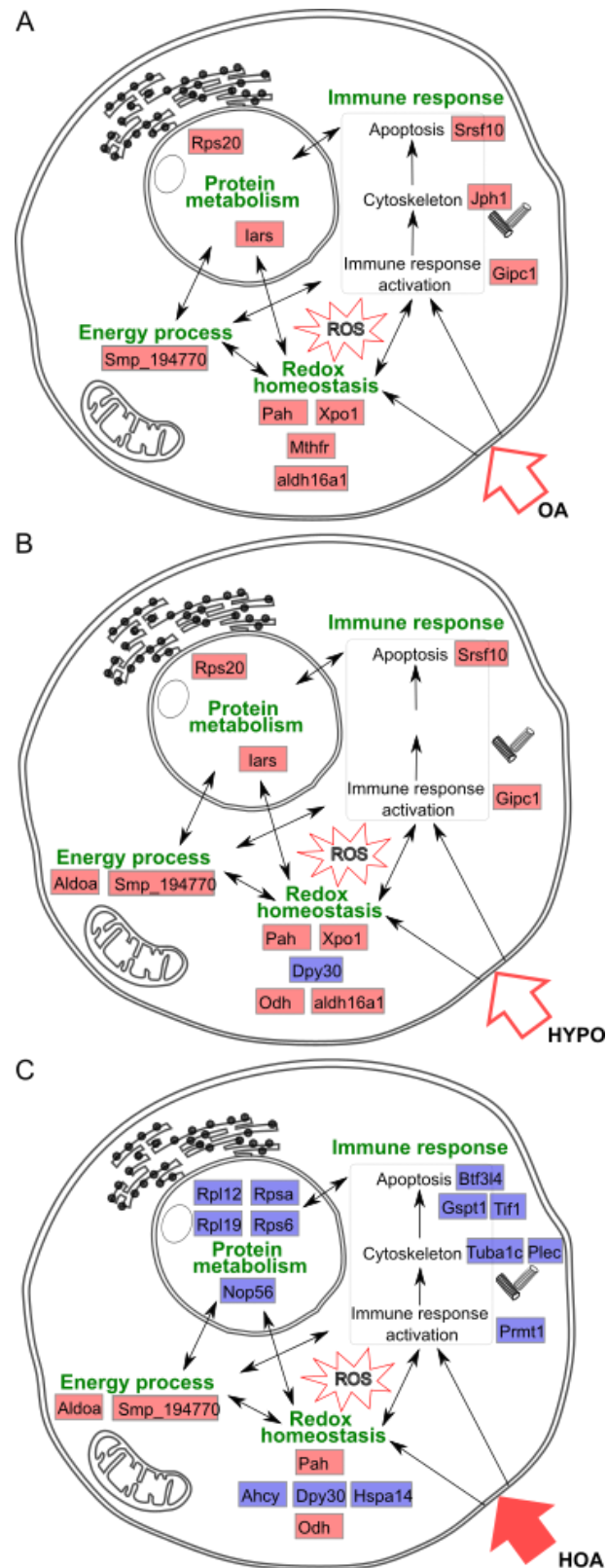


FIG. 5.2: Schematic representation of redox homeostasis, energy production, protein metabolism and immune system in (A) OA, (B) HYPO and (C) HOA conditions in the proteomic profile of *A. purpuratus*. Red and blue boxes color mean accumulation and deregulation of proteins, respectively. The figure reveals the importance of redox homeostasis in all treatment and the different strategy in HOA.

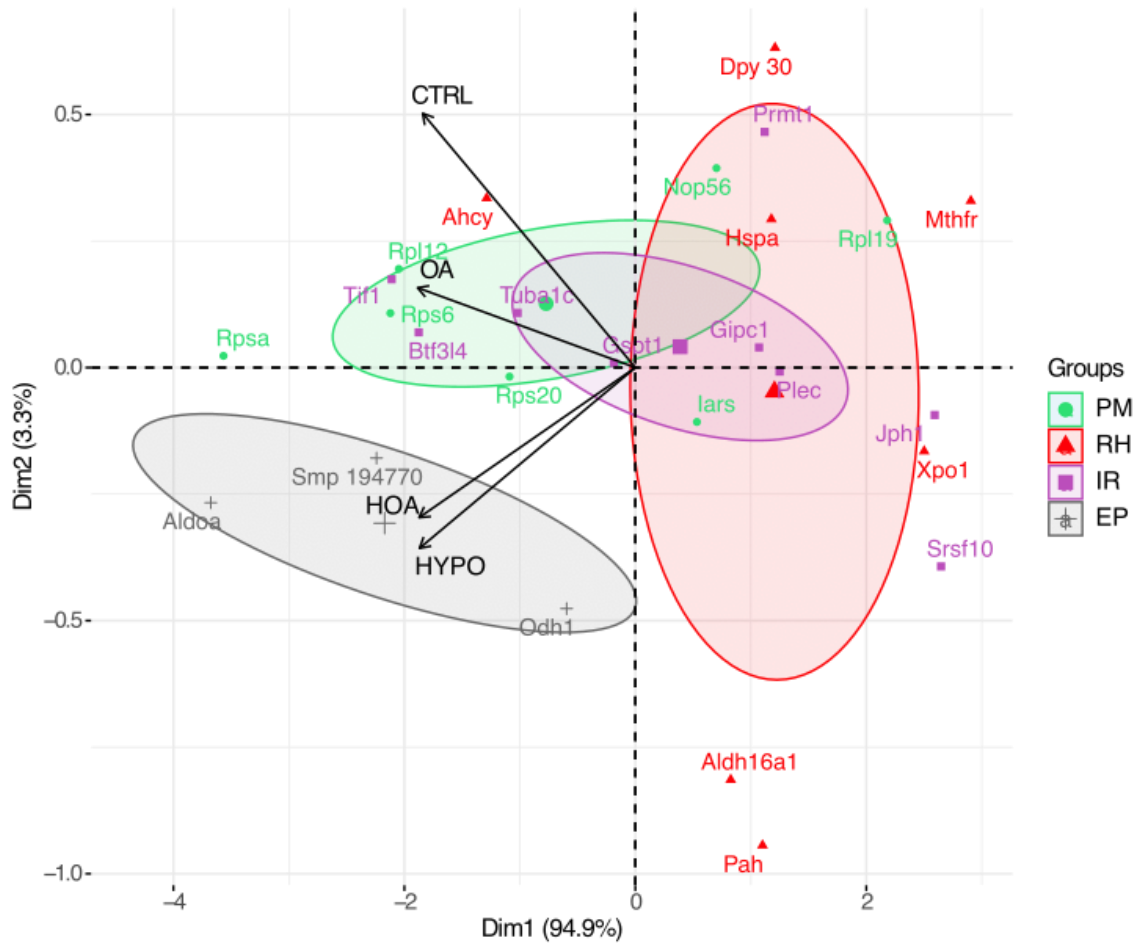


FIG. 5.3: Principal component analysis of the average of the 27 DE log₂-protein intensities in the HYPO, OA, HOA and CTRL treatments. Biplots represent the two first principal components with 94.9 and 3.3% of the total variability explained. The proteins are grouped according to the functional role proposed: protein metabolism (PM), redox homeostasis (RH), immune response (IR) and energy production (EP). Ellipses indicate 95% confidence limits of the latter grouping.

High demand of energy could be linked to the synthesis of proteins that help maintain homeostasis in the organism (See Fig. 5.2), which is consequent with results presented by Artigaud et al. (2015) in the scallop *P. maximus* exposed to hypoxia. In fact, proteome of bivalves exposed to single hypoxia showed increased mitochondrial energy production to maintain cellular homeostasis, by accumulating antioxidants, as response to oxidative stress (Thompson et al., 2016 ; Sokolov et al., 2019 ; Andreyeva et al., 2021). The accumulation of Pah, Xpo1 and Aldh16a1, could be linked to their role in redox homeostasis during hypoxia. It coincides with the results in other studies in bivalves after environmental stressors (Nesto et al., 2004 ; Ji et al., 2014). In the literature, Pah has an important antioxidant role as a ROS scavenger (Calvo et al., 2008) and osmotic regulation as response to the organic pollutants (Ji et al., 2014). Xpo1 is a transport factor responsible for the nuclear export of several hundred proteins and RNA substrates, besides control of gene expression through regulated mRNA stability. Elevated levels of Xpo1 and recurrent mutations have been linked to advanced disease stage and poor survival in humans (Kim et al., 2022). This protein exhibited a pronounced accumulation in the single treatments,

suggesting potentially more harmful effects when compared to the combined stress treatment, particularly evident in hypoxia due to the intense accumulation of Aldh161 in the single treatments, specially in hypoxia. This protein is linked to detoxification processes (Ahmed Laskar and Younus, 2019) : in zebrafish *Danio rerio* is critical to maintain the LC-pufa homeostasis through fatty acid metabolism degradation (Zhao et al., 2020) and has been used as a biochemical marker to evaluate the biological responses of the mussel *M. galloprovincialis* to natural and anthropic stresses (Nesto et al., 2004).

Proteins accumulated in HYPO are connected between themselves (See Fig. 5.1B) and are associated with the synthesis of amino-acids (Rps20 and lars) and may be closely related with the accumulation of proteins linked to energy production, homeostasis and immune response. Rps20's accumulation has been reported before against oxidative stress in zebra mussel (*Dreissena polymorpha*) exposed to microplastics (Magni et al., 2019) and in *Ruditapes philippinarum* exposed to copper (Volland et al., 2017). lars, linked with the tRNA binding and metabolism related pathways, were found in the sea cucumber *Apostichopus japonicus* as response related to immune defense at bacterial infection (*Vibrio splendidus*) (Zheng et al., 2019). Excessive pyruvate and lars will promote the biosynthesis of leucine, isoleucine and valine, through resulting in increased levels of glucose and branched amino acids (Zhang et al., 2014). On the other hand, accumulation of proteins linked to immune response (Gipc1, Jph1 and Srsf10) suggests that long exposure to hypoxia may induce activation of apoptosis to palliate the cellular damage produced in the mitochondria. Sokolov et al. (2019) suggest that hypoxia-tolerant marine bivalve have high antioxidant defenses in anticipation of oxidative stress, and high activity of mitochondrial proteases involved in degradation of damaged mitochondrial proteins. PdZ domain-containing protein Gipc1 protein has been involved in the host response to White-spot syndrome virus (WSSV) infection in the claw crayfish *Cherax quadricarinatus* (Jeswin et al., 2016). Meanwhile, Srsf10 is related with ribonucleoprotein complex biogenesis and complex assembly and negative regulation of gene expression, and after DNA damage controls splicing decisions in transcripts encoding components involved in DNA repair, cell-cycle control, and apoptosis (Shkreta et al., 2016).

5.4.2 Mechanisms to respond reduced pH

Reduced pH (and accompanying CO₂ levels) have an influence on the physiology of marine organisms through acid-base imbalance (i.e. extracellular acidosis) and reduced oxygen transport capacity (Fabry et al., 2008 ; Dupont and Thorndyke, 2012). In the oysters *C. gigas* (Wei et al., 2015), *Saccostrea glomerata* (Thompson et al., 2016) and the geoduck *Panopea generosa* (Timmins-Schiffman et al., 2020), the exposition to reduced pH causes an elevated energetic demand, resulting in disturbances in carbohydrate metabolism, cell growth, cytoskeleton dysfunction, protein synthesis and the extracellular matrix. Our results showed a broad accumulation of proteins that interact with each other (Fig. 5.1) ; this is consistent with the disturbances recorded above in the cell, in fact, this treatment was closely related to the role of protein synthesis (See PCA, Fig. 5.3).

Mthfr and Jph1 are significantly accumulated in OA treatment compared to HYPO (Fig. 5.2, Table 5.1). Mthfr accumulation may suggest the need for the individuals to balance out excessive ROS and acid-base disturbance produced

by the reduced pH. Wang et al. (2016) presented Mthfr as a major detoxification enzyme, overexpressed in the hard clam *Mercenaria mercenaria* after Quahog Parasite Unknown infection Wang et al. (2016). For its part, Junctophilin (Jph, a structural protein), is regarded as key regulators of many cellular processes, ranging from proper cell division to maintaining cell shape and cytoskeletal remodeling (Wang et al., 2016). Jph1 anchors the endo/sarcoplasmic reticulum to the plasma membrane and may be also involved in regulating Ca^{2+} homeostasis (Golini et al., 2011). Despite Jph1 being a protein linked with immune response, a study shows that OA could depress the cellular immune response through the decrease in phagocyte numbers in sea urchins (Dupont and Thorndyke, 2012). On the other hand and similarly to HYPO treatment, OA shows an accumulation of Pah, Xpo1 and Aldh16a1, which along with Mthfr can play an important role in redox homeostasis. The presence of these proteins with the other accumulated proteins (Smp 194770, Rps20, Iars, Srsf10, Jph1 and Gipc1 ; Figure 1.2, Table 5.1), shows an active metabolism of 51-days survivors which may help *A. purpuratus* cope reduced pH. The higher mortality observed in this treatment is consistent with findings from a study on tubeworms' proteomic profiles, which suggests that ocean acidification (OA) stress alone had a more significant impact than hypoxia stress alone (Mukherjee et al., 2013).

5.4.3 Mechanisms to combined hypoxia and reduced pH

The study delves into the protein profiles of organisms subjected to combined environmental stressors, highlighting distinct mechanisms compared to single stressors. Hypoxia and reduced pH alone prompted a broad deregulation of proteins (Table 5.1, Fig. 5.2), affecting protein synthesis and immune response. This behavior has been found in oyster larvae, suggesting a link between physiological suppression and protein down-regulation (i.e. depression of metabolic genes expression) under OA (Dineshram et al., 2012). During periods of oxygen limitation, organisms often suppress ATP-demanding processes, such as protein synthesis, to extend survival (Hand, 1991). This strategy may represent an acclimation mechanism to hypoxia and reduced pH in *Argopecten purpuratus*, reflected in lower mortality rates in the combined stress treatment compared to individual stressors. Similar findings of an antagonist effect have been reported in tubeworms, where hypoxia appeared to counteract the negative effects of reduced pH (Mukherjee et al., 2013). However, the reduced protein synthesis associated with acclimation can hinder growth and reproduction, impacting species survival in the long term (Fabry et al., 2008).

Proteins linked to energy production and homeostasis were accumulated in the combined hypoxia and reduced pH treatment, including Odh and Pah. Odh plays a role in energy metabolism and homeostasis by buffering toxic end-products of anaerobic respiration (Seibel et al., 2000). Pah acts as an antioxidant and contributes to homeostasis (Calvo et al., 2008 ; Ji et al., 2014). According to Sui et al. (2017), both stressors induced antioxidant defense in the mussel *M. coruscus*. These proteins are signs of acclimation to long-term hypoxia and reduced pH in survivor scallops, potentially driven by genetic variability (Acosta-Jofré et al., 2020) and adaptation to this high variable environment. However, smaller individuals (as used in this study) may have higher hypoxia tolerance (Cueto-Vega et al., 2021), therefore the influence of individual size on stress response should be taken into account.

Other proteins important for the oxidative stress response like Dpy30, Hspa14, and Ahcy (Kregel, 2002 ; Jeswin et al., 2016 ; Thompson et al., 2018 ; Sarkar et al., 2020) were deregulated under combined stress. Dpy30 is responsible for molecular transport, and its deregulation may disrupt metabolic pathways linked to hematological diseases (Yang et al., 2016 ; Xu et al., 2009). Hspa14 has a protective role against oxidative stress, via reducing the oxy-radical accumulation and stabilizing mitochondrial functions (Seo et al., 2008). Whereas, Ahcy and Prmt are linked to the amino-acid biosynthesis pathway and their reduction due to hypoxia may indicate an increase in adenosine metabolism (Kobayashi et al., 2000). Proteins associated with protein synthesis (Rpsa, Rps6, Rpl12, Rpl19, and Nop56) and immune response (Prmt1, Tuba1c, Plec, Btf3l4, Gspt1, and Tif1) were also deregulated, suggesting potential metabolic disruption in the combined treatment. Notably, some proteins indicate poor immune response and apoptosis regulation. Prmt1's over-expression in immune response and inflammation-related pathways may reveal the metabolic consequences of its deficiency after exposure to environmental stressors (Xiong et al., 2020). Tuba1c is a protein involved in microtubule-based process and GTPase activity, its participation was important after viral infection in the mussel *Perna viridis* (He et al., 2019), its deficiency may also indicate a poor immune response. Meanwhile, plectin binds to various cytoskeletal proteins including microtubules and intermediates filaments and regulates cellular survival, growth, and polarization signaling pathways (Wiche et al., 2015 ; Martin et al., 2016). Gspt1 is one inhibitor of inhibitors of apoptosis (IAP)-binding proteins released from the mitochondria of cells during apoptosis and regulate apoptosis by liberating caspases from IAP inhibition (Hegde et al., 2003), this protein may control the termination of translation, protein biosynthesis and regulation of gene expression. By his part, Btf3l4 and Tif1 are proteins involved in cell-cycle regulation and apoptosis-related pathways ((Zhao et al., 2020), and were found differential expressed in studies under hypoxia or pollutants in bivalves (David et al., 2005 ; Sanson et al., 2020).

5.5 Conclusions

In summary, the proteomic profiles of *A. purpuratus* under prolonged stressful conditions of hypoxia and acidification reveal distinct response mechanisms between single and combined stress treatments after 51 days of exposure. The accumulated proteins were categorized into processes related to redox homeostasis, immune response, energy production, and protein metabolism. In the single stress treatments (HYPO and OA), proteins associated with all four functions mentioned above were found to accumulate. However, in the combined treatment (HOA), metabolic depression appeared to be employed to minimize energy expenditure, with a priority on maintaining energy production and homeostasis. Consequently, several proteins linked to these functions (Pah, Aldoa, Smp 194770, and Odh) were accumulated in all treatments, suggesting their potential use as indicators of acclimation and stress markers in this species. Evaluating the impacts of multiple environmental stressors, which is a more realistic scenario than considering them individually, is crucial for predicting the effects of environmental changes on coastal organisms. Our findings provide valuable insights into the potential combined effects of reduced pH and dissolved oxygen levels on marine bivalves.

Chapter 6

Synthesis

6.1 Laboratory vs field observations

Ecophysiology aims at understanding how organisms respond to their environment. This discipline uses different experimental approaches to assess organisms' response, such as laboratory, field and natural experiments (Spicer, 2014). In a laboratory experiment, we can control the intensity of environmental stressors, test them separately or jointly, and measure the organism's response to these constraining variables. Laboratory experiments have the advantages of simplicity, minimization of extraneous variables, easy replication, and can lead to causal conclusions ; However, it is a contrived experience for the organisms and it may have laboratory effects (Spicer, 2014). Natural experiments are quasi experiments performed in the field, with little human intervention, that take advantage of the natural environmental variations. They are ecologically realistic, but due to extraneous variables and unique events, it is not possible to draw a causal conclusion (Spicer, 2014). Field experiments are in between ; experimenters have a certain level of control with more or less ecological realism, and they can draw some causal conclusions about the stressor(s). High-frequency monitoring presented in Chapter 2 during four scallops mortality events that occurred in Paracas and Sechura Bays can be considered as "natural experiments". They reveal one of the problems of natural experiments when working with extreme events : each event was unique (in terms of temperature variability, duration and intensity of hypoxia) ; so, mortality causes related to severe hypoxia were evident but hardly testable. For example, before the massive mortality records of 2015 and 2019 in Sechura Bay, scallops were exposed for up to 3.9 and 12 days under continuous severe hypoxia (5% DO saturation) with temperatures ranging between 17 and 21 °C (see Chapter 2). In Paracas, the scallop mortality events of 2013 and 2015 occurred after 6.8 to 7.6 days of severe hypoxia on the seabed, with pulses of temperatures ranging between 15 and 24 °C. Thus, one would need to study numerous case studies of stress and mortality events both within each bay and between bays in order to be able to draw general trends, patterns and thresholds regarding the effects of what will always be combined stressors. The latter imply also that environmental variables which may affect scallop responses other than temperature and oxygen, such as ocean acidification, food, the presence of toxic algae or H₂S, microbial proliferation, etc., be monitored in natural and field experiments. Furthermore, patterns or thresholds registered for a given bay will not necessarily be the same as those observed or estimated in another area with different combinations or thresholds of stressors.

On the other hand, most of the work presented in this thesis was based on laboratory experiments (Chapters 2, 3 and 4) that allowed isolating variables and exploring the effects of hypoxia and acidification on the physiology of the Peruvian scallop. We assume that the negative effects recorded due to the stressor levels chosen were higher on survival (and respiration rates) than those generated by laboratory maintenance. Indeed, the negative effects of the stressor variables on scallop physiology are consistent with results from field experiments

performed on the species Aguirre-Velarde (2016) ; Ramajo et al. (2020). The apparently low effect of laboratory-exposure-related stress suggests a certain degree of replicability when individuals face hypoxia and acidification. This may validate our findings about the physiological response of the species to the environmental stressors tested.

6.2 Does scallop size matter?

The exponent of the relationship between weight and respiration rates was 0.75 (Chapter 3), which suggests that greater weight demands more oxygen consumption. The oxygen demand is proportional to two components : surface (L^2) and volume (L^3 , or weight when assuming an isometric growth) ; this may be the reason why the exponent of the weight - respiration rate relationship has an exponent between 2/3 to 1 (Kooijman and Kooijman, 2010). The surface-to-volume ratio (S-V) hypothesis states that smaller organisms may be more tolerant to hypoxia than larger organisms : when an organism is small, it has a higher surface-to-volume ratio because its gills are relatively large compared to its body weight. Now, the capacity to extract oxygen from the water is proportional to the surface (L^2 , of the gill or of the body), while the oxygen demand has a component which is proportional to the weight (L^3). Then, as the individuals grow, their tolerance to hypoxia is reduced due to lower surface-to-volume ratios, probably resulting in a greater energy demand to maintain aerobic metabolism. The median lethal time in experimental conditions close to anoxia (1% DO saturation ; Chapter 2), the respiratory response in progressive hypoxia, and the recovery after prolonged exposure to severe hypoxia (Chapter 3) tend to validate the S-V hypothesis because smaller-sized *A. purpuratus* individuals : (1) survive longer under severe hypoxia, (2) have a greater oxy-regulation capacity, and (3) have a greater recovery capacity after a short severe hypoxia exposure than larger ones.

The trend of scallop (*A. purpuratus*) greater ability to cope with hypoxia in the smaller individuals cannot be generalized to other bivalves because of particular physiological strategies depending on species. In Chapters 2 and 3, we revised coincidences and differences with other studies carried out on bivalves ; in summary, the fact that a species is more tolerant to hypoxia than another one and the positive or negative size-dependent relationship would be related to the habitat where it lives and to the intensity of the hypoxia. A hypoxia-tolerant mytilid species living in the intertidal zone may have different responses than subtidal scallops, due mainly to the fact that the former is naturally exposed daily to longer hours of anoxia. Species facing daily cyclical hypoxia, such as *A. purpuratus* (Aguirre-Velarde et al., 2016, 2019b), may have a great oxy-regulatory capacity, but little tolerance to anoxia. On the other hand, a species adapted to life in anoxic environments or to endure aerial exposure, has a tendency to be oxy-conformant (Hicks and McMahon, 2002), can use efficiently the anaerobic metabolism (Hicks and McMahon, 2005), and reduce its energy expenses (Storey and Storey, 1990). These patterns would allow oxy-conformers to survive more days under anoxia than oxy-regulators. Using anaerobic metabolism requires having greater energy reserves, which is the case in large animals compared with smaller ones ; the former can survive longer (see discussion in Chapter 2 and 3). Another explanation for different ability to cope with hypoxia between species may be related to the foreseeability or not of hypoxia exposure (e.g. tide-related natural aerial exposure, daily cycles). If the hypoxia exposure is short and predictable, the oxy-conformant strategy might be the best one because individuals save

energy for homeostasis, reduce energy expenses, and instead switch rapidly to an anaerobic metabolism. At some point, reoxygenation can help to transform fermentation end-products. By contrast, if hypoxic crises are unpredictable and last longer, the oxy-regulatory strategy would be better when considering the delay to start anaerobic metabolism and the formation of toxic metabolic end-products.

Lower tolerance to hypoxia in larger bivalves may be also related to their reproductive effort during maturation and/or spawning. For example, mature and post-spawn scallops are more vulnerable to hypoxia than immature scallops Brokordt et al. (2015) ; Salgado-García (2020). Although the effects of reproduction on hypoxia tolerance were not analyzed, the former could be real when considering the high variability in respiration rates, P_{cO_2} and oxygen regulation percentage of the large scallops exposed to progressive hypoxia (Chapter 3). Even so, the effect of size on hypoxia tolerance was apparently greater than the effect of the reproductive cycle. Higher condition index enhances survival after extreme hypoxia exposure (Chapter 2) ; it has been shown that greater energy reserves contribute to a high tolerance to anoxia in some marine invertebrates because reserves are used as energy source during anaerobic metabolism (de Zwaan and Zandee, 1972).

6.3 Experimental design should consider tolerance thresholds and exposure time

It is important to define tolerance thresholds for the environmental drivers of a species because they are related to higher levels in ecology : thresholds may link the individual response from populations to communities. Thresholds may define the abundance, distribution, and boundaries of populations, and their sustainability (or not, which signifies local extinction) in given habitats. Currently, this topic becomes concerning due to the effect of climate changes on marine organisms and their ecosystems (Pörtner et al., 2014). To define scallop tolerance thresholds for hypoxia and acidification, we could use the OCLTT approach defined by Pörtner and Lannig (2009, ; “Oxygen and Capacity Limited Thermal Tolerance”) with four thresholds : optimal levels (where growth, reproduction and exercise are optimal), pejus levels (getting worse, in which stress tolerance is limited by exposure time), critical levels (when oxygen becomes insufficient and individuals begin to use anaerobic metabolism), and lethal levels (which produce the death of the individuals in the short term). Throughout this thesis, working with different levels of dissolved oxygen saturation (Chapters 2 and 3) allowed identifying critical levels of oxygen (ranging from 12.5 to 24.9% DO saturation) and lethal level (~ 1% DO saturation). Lethal level produced mortality from the second day on (Chapter 2). Severe hypoxia (5% DO saturation, Chapter 3) could be also lethal because an oxygen debt was observed in both size batches after 12-hour exposures. In intertidal bivalves, the oxygen debt payment depends on the time of exposure : the longer the exposure to anoxia, the higher the magnitude of the oxygen debt (Shick et al., 1988). If the exposure is long enough, oxygen debt can accumulate causing a depletion in the energy reserves and ultimately the death of the individuals. In Peruvian bays, such as Paracas, continuous severe (< 5% DO saturation) and moderate hypoxia (< 24% DO saturation) can last up to 4 and 13 days, respectively (Chapter 2). Deepening in the energy investment at sub-lethal levels should reveal information

about the last effort carried out by the organisms to try to maintain homeostasis. With respect to future ecophysiological studies, it is recommended to perform a lethal time experiment under severe hypoxia with a monitoring of the evolution of energetic metabolism throughout the experiment (energy expenditure, metabolic pathways used according to time, energy balance, abundance and toxicity of metabolic end-products...).

Pejus levels were partially considered in this thesis because we worked at 20% DO saturation, which is higher than the PO (close to 13% DO saturation) estimated for the scallop smaller size (Chapter 3). This is consistent with the slightly negative effects produced by the oxygen stressor after the first 14 days, such as a decrease in clearance and calcification rates, and relatively low mortality rate (<10%, Chapter 4). This trend was the same for the single acidification and the combined hypoxia and acidification treatments. The effect of stressor on growth and reproduction (considering the gonadosomatic index) was little since all the stress treatments and the control showed quite similar measurements. A DEB (Dynamic Energy budget) modelization performed on the scallop species suggested that a decrease in growth and reproduction after chronic and severe hypoxic periods (observed in the field) may be explained by a decrease in both assimilation and reserve mobilization (Aguirre-Velarde et al., 2019a). The reduced clearance rate found in stress treatments but relatively good growth may indicate some kind of optimization in the food assimilation, absorption or another related process within the digestive system. This optimization in digestive processes could signify energy saving for other important metabolic processes and may help to cope with homeostasis. It is suggested deeper knowledge of the mechanisms underlying feeding processes in response to hypoxia and acidification. For this purpose, disciplines such as histology, biochemistry and proteomics could be useful. On the other hand, the decreases in calcification rates would be related to the decreases in the clearance rates ; they may have negative effects on the scallop mobility and escape response as suggested by other authors (Kraffe et al., 2008 ; Brokordt et al., 2013).

The negative effects on clearance and calcification rates continued after 51 days ; there was also a significant decrease in survival in all stress treatments (Chapter 4). Nevertheless, the growth rates were quite similar in all treatments (stress and control treatments) and were consistent with VBGF growth models for the species. Additionally, measurements related to reproduction (GSI and histological analysis, oocyte types frequency and size) revealed a gonad maturation only in the stress treatments. A prolonged exposure to the stress condition did generate changes in the mantle proteome between the single stress and the combined treatments ; an accumulation of several proteins was observed in the single stress treatments while few proteins were accumulated and several were deregulated in the combined stress treatment. The proteins that mostly accumulate under stress treatments were related to maintenance of homeostasis, energy production, protein synthesis and immune response. Accumulation of octopine was observed in the mantle of individuals exposed to the hypoxia treatments (Chapter 5), indicating the use of anaerobic pathways. Most likely, under chronic exposure to hypoxia, both aerobic and anaerobic metabolisms may be used (Salgado-García a, 2020). As described above, prolonged pejus levels (of environmental stressors) should generate negative effects on organisms ; these effects are often underestimated, but they may play an important role in constraining the life traits of the species. Clearly, the medium-

and long-term time scales must be taken into account in ecophysiological studies ; in the next section, some proposals are made on what should be taken into account in future laboratory experiments on scallop responses to stress conditions.

6.4 Physiological trade-offs under combined hypoxia and acidification

Despite the limitations of the study and the intra-treatment variability, the lower survival but higher reproductive investment in the acidic treatment suggest an apparent energetic trade-off between homeostasis and maturation. In the single OA treatment, and despite lowest survival rate, the individuals exhibited quite similar growth, gonad maturation than other stress treatments (Chapter 4, Fig. 4.3), and an over-regulation of proteins (Chapter 5, Fig. 5.1), which suggest an active homeostasis process and early maturation in survivors. The scallops exposed to the combined stress treatment had a higher survival rate, quite similar growth and apparent earlier maturation (see oocytes frequencies, Chapter 4, Fig. 4.3) than the ones exposed to single stress treatments. However, individuals from the combined treatment presented fewer oogonia (immature oocytes, Chapter 4) than the ones exposed to other conditions, as well as a de-regulation of several proteins (Chapter 5) probably indicating a depletion of energy. The earlier maturation under stress condition might be explained by a possible aging process : the oxidative stress caused by hypoxia and acidification would produce early ripening. Pauly (2021) proposed the gill-oxygen limitation theory which uses the same argument as the surface-to-volume ratio, but applied to various other physiological responses. A lower oxygen relative supply would induce sexual maturation before a reduction and cessation of growth. Early maturation may suggest a reduction in the maximum length. As the energy demand for the metabolism maintenance is higher in large individuals and environmental stressors induce greater energy costs to maintain homeostasis, these costs will be higher in larger scallops. Being small implies lower cost for somatic and maturity maintenance (van der Veer et al., 2003). Now, early reproduction has some disadvantages because it implies smaller gonads and low reproductive outputs that may impair the reproductive success (Haag, 2013). Smaller individuals may also be more susceptible to predation, particularly if they have thinner and weaker shells (Gaylord et al., 2011). Stress on the broodstock probably affects individual energy reserves and may play an important role in larval survival and performance. Low maternal investment may affect the larval development among benthic marine invertebrates (Pechenik, 1999). Conversely, acclimatized survivors could inherit to their offspring the features to cope with the stressors encountered (called a transgenerational acclimatization effect, Goncalves et al., 2017). In the case of the combined stress treatment of our study, the lower frequency of oogonia in the gonads is rare because this species exhibits a continuous production of gametes along the year (Cantillanez et al., 2005). Low production of oogonia and de-regulation of several proteins in the combined stress treatment would indicate that early maturation was unique and probably the last maturation process suggesting an 'emergency' reproductive strategy.

Both hypoxia and acidification cause internal acidosis (Storey and Storey, 2004 ; Pörtner et al., 2004), but the external rise in $p\text{CO}_2$ and drop in pH (due to acidification) require additional energy expenses for pH and ion regulation,

respiration and calcification rates. They may affect blood oxygen transport and mitochondrial processes regulating homeostasis and energy balance of the organisms (Duncan et al., 1994 ; Pörtner, 2008 ; Pörtner et al., 2017). It is likely that low pH plays a more significant role than hypoxia in detriment to the steady state of individuals (due to the lowest survival ; see Chapter 4). The higher survival in the combined stress treatment suggests an antagonistic effect of the combination of hypoxia and acidification, probably related to the early onset of oxy-conformity, reducing energy costs from the first weeks (see clearance rates at 14 days ; Chapter 4, Table 4.2). As both variables occur simultaneously in its natural environment, the scallop response would be better when both variables occur together, compared with single stress. Taking into account the probable emergency reproductive process despite higher survival in this treatment, the most deleterious environmental stressor on growth or reproduction remains to be determined. Stress-related signals in life traits were weak after 51 days ; one reason for this would be that the experimental time was short compared with the scallop lifespan (cultured scallops may live approx. 1.5 - 2 years). To highlight such signals in the life traits (long term growth, maturity, reproduction success...) and better understand the physiological trade-off in the organisms under environmental stressors (hypoxia and acidification), it is recommended : (1) performing a similar experiment (using pejus thresholds), but on longer time, even though this could be a contrived experience inducing stress linked to laboratory exposure ; (2) performing a short experiment using critical levels and considering the individual size. In this case, a sampling frequency of less than a week is suggested ; or (3) modeling the life trait response by using a DEB model, applied before on *A. purpuratus* by Aguirre-Velarde et al. (2019a). This model tries to explain, and predict how the environment affects life traits of individuals at longer time scales, which is useful for ecological and evolutionary purposes. DEB modeling may help fill this gap due to the short-time experience, and allows to predict how repeated stress (due to chronic exposition) may affect the scallop life traits. Since this type of model can be only developed based on the knowledge of metabolic processes, the latter need to be better understood. For this reason, it is also suggested deepening in the underlying metabolic processes by the use of cellular (energy metabolism) and molecular analysis (use of -omics analysis).

The experimental work carried out in this PhD thesis was performed at stable temperature conditions (16 °C) taking into account that the oxygen critical values (~20% DO saturation) in this scallop species vary slightly between 16 and 23 °C (Aguirre-Velarde et al., 2016). Critical values of pH have not been defined yet, thus dedicated studies are recommended. A respirometric study under progressive acidification could be useful because the reduction in oxygen consumption could give some evidence of limiting process(es) at low pH levels. Besides, evaluating the critical and pejus thresholds of other environmental stressors (such as warming or abrupt changes in temperature, sulfur, among others) is also recommended. A continuous environmental monitoring in Peruvian coastal bays and the estimation of the stress thresholds will be necessary to better understand the stress effects on the scallop stocks in its northernmost ecological niches.

Shallow waters from the Humboldt Current System have been shown to exhibit highly variable temperature, oxygen concentration and pH, which can change abruptly due to intense upwellings, incoming oceanic water masses, events such as El Niño (which produce river water inflows and water column

stratification) or La Niña (upwelling intensification), among others. The prolonged exposure to moderate hypoxia and ocean acidification used in the present study may not be a realistic actual scenario (moderate hypoxia lasted for a maximum of 13 days between 2012 and 2019). It may be another story when moderate thresholds will become severe or will last longer, e.g. during extreme events, or in a future scenario of deoxygenation and acidification in coastal EBUS (see review of Chapter 1, section 1.1). Ecophysiological approaches developed within this work at the organism, tissue and molecular levels showed that the negative impacts of hypoxia and acidification induced some physiological trade-offs between homeostasis and early maturation. It was also found that individual size plays an important role in scallop ability to acclimatize to hypoxia. The results also suggested that the acclimatization of the species to environmental stressors depended on the size of the individuals, and the intensity and time of exposure to both hypoxia and acidification. In a global change scenario, the warming of the sea coupled with intense stratification in the water column could cause a selection towards small size in this scallop species (Gardner et al., 2011). Ecophysiological studies from different approaches (physiological rates, histological and proteomic analyses, among others) complemented each other and helped to better evaluate and understand how the Peruvian scallop responds to current and future environmental changes and how organisms' response may affect its ecological niche.

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Titre : Effet de l'hypoxie et de l'acidification sur la physiologie du pétoncle péruvien *Argopecten purpuratus* (L.)

Mots clés : *Argopecten purpuratus*, réponse physiologique, hypoxie, acidification.

Résumé : L'hypoxie et l'acidification, stress environnementaux majeurs, affectent les populations marines du système du courant de Humboldt le long des côtes du Pérou et du Chili, provoquant des épisodes de mortalité massive, notamment chez le pétoncle péruvien *Argopecten purpuratus*. Notre recherche avait trois objectifs principaux. Tout d'abord, caractériser les conditions d'hypoxie dans les baies de Paracas et Sechura. Ces baies ont connu des périodes prolongées d'hypoxie modérée et sévère, durant plusieurs jours pendant la période d'étude. Deuxièmement, examiner l'influence de la taille des pétoncles sur leur tolérance à l'hypoxie en laboratoire (survie, régulation de l'oxygène et récupération post-stress). Les pétoncles les plus petits ont montré une meilleure tolérance à l'hypoxie. Enfin, étudier les réponses physiologiques et protéomiques des pétoncles juvéniles à l'hypoxie et à l'acidification à moyen terme.

Une exposition prolongée à ces stress a entraîné une réduction des taux de filtration et de calcification, une maturation accrue, des altérations de la survie, et des modèles distincts de régulation et de dérégulation protéiques. Dans le traitement combinant les stress, des protéines liées à l'immunité et au métabolisme protéique ont été dérégulées, tandis que peu de protéines liées à l'énergie et à l'homéostasie ont été accumulées. Ces résultats suggèrent un compromis énergétique entre homéostasie et maturation, ce qui pourrait affecter la croissance et la reproduction. Dans des scénarios de changement climatique et d'événements extrêmes, la capacité accrue des individus de petite taille à résister aux stress environnementaux pourrait favoriser une sélection en faveur des individus à faible croissance.

Title : Effect of hypoxia and acidification on the physiology of the Peruvian scallop *Argopecten purpuratus* (L.)

Keywords : *Argopecten purpuratus*, physiological response, hypoxia, acidification.

Abstract : Hypoxia and acidification are significant environmental stressors affecting marine populations in the Humboldt Current System along Peru and Chile's coastlines. Over the past decade, the region has experienced several mass mortality events, particularly among benthic organisms like *Argopecten purpuratus*, often linked to extreme hypoxia events. Our research pursued three primary objectives. Firstly, it characterized the environmental conditions associated with hypoxia in two Peruvian bays, Paracas and Sechura. These bays exhibited prolonged moderate and severe hypoxia events, with durations of several days. Secondly, we examined how individual scallop size influenced survival, oxygen regulation, and recovery when exposed to extreme hypoxia in laboratory settings. Smaller scallops displayed higher hypoxia tolerance. Lastly, we investigated

the physiological and proteomic responses of juvenile scallops to medium-term exposure to hypoxia and acidification. Prolonged exposure to these stressors resulted in reduced filtration and calcification rates, stimulated maturation, lower survival rates, and distinct protein accumulation and deregulation patterns. In the combined stress treatment, several immune and protein metabolism-related proteins were deregulated, while only a few energy- and homeostasis-related proteins were accumulated. These findings imply an apparent energy trade-off between maintaining homeostasis and reproductive maturation, potentially affecting growth and reproduction. In extreme events and amidst climate change scenarios, enhanced ability of smaller individuals to withstand environmental stressors could drive a selection process favoring slow-growth individuals.